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13. ABSTRACT *(Maximum 200 words)*

This is the final report for the project. We had previously determined that the NAT2 slow acetylator genotype and cigarette smoking is a risk factor for breast cancer. In this project, we evaluated the relationship of several genetic variants and breast cancer risk. MnSOD2 was a risk factor, that was increased by diets low in antioxidants. We did not find that NAT2 and meat consumption was a risk factor. Other genetic analyses are completed for *MEH3, MEH4, CYP2D6, GSTM1, GST-T and CYP1A1*. We have been validating a p53 mutational spectra detection technology using the Affymetrix gene chip array. This is going well and more than 300 blocks have been received for analysis, with another 200 to be delivered. The spectra in all these blocks will be completed with additional funding from the NCI Intramural program (about 100 tumors are completed to date). To corroborate the epidemiological data, 53 breast cell strains have been established and metabolism is being studied in relation to genotypes. Methodological conditions are being addressed. We are also developing a new carcinogen-DNA adduct methodology for use in breast tissues. The preliminary data for this was supported by this grant. We also have been studying smoking behavior and addiction, where, genetic polymorphisms in dopamine receptors and the dopamine transporter gene were addiction risk factors. This project resulted in 19 publications to date.

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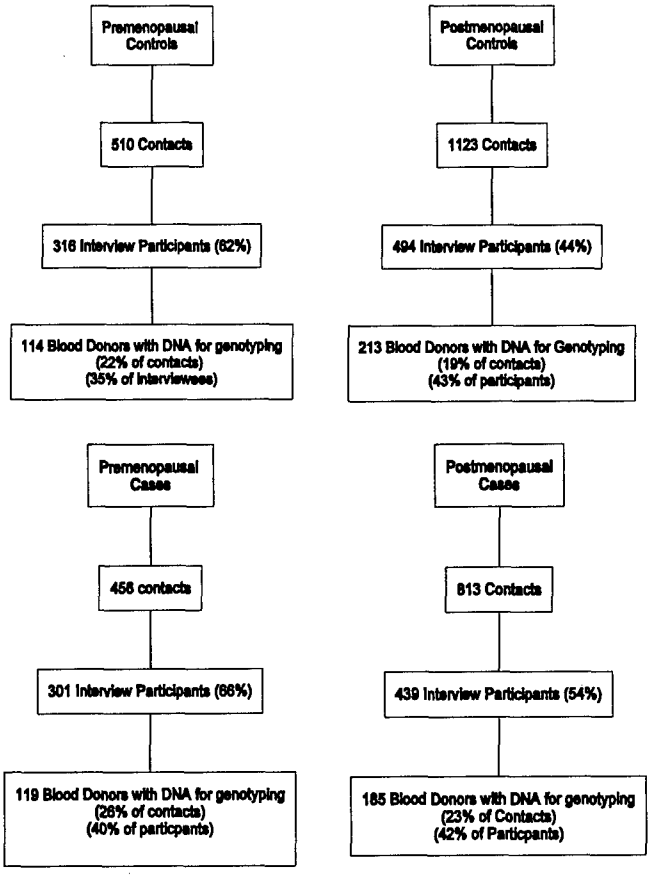
INTRODUCTION

The first goal of this project is to determine the frequency of genetic polymorphisms for carcinogen metabolism and the p53 mutational spectra in a previously conducted breast cancer study. This study was designed to assess nutritional risk factors, seeking to identify risk factors related to inheritable susceptibilities and chemical etiologies. The workscope of this DOD grant was subsequently expanded to include the same goals, but for other epidemiological studies of breast cancer, and to perform studies of breast metabolism, p53 and smoking (including smoking cessation; a copy of the letter requesting the approval is shown in Appendix A). The DOD grant allows us to examine a variety of risk factors (hormonal and non-hormonal; environment and diet; carcinogens and anticarcinogens) in relationship to p53 mutations and breast cancer with genetic polymorphisms as effect modifiers. The frequency of genetic polymorphisms themselves in relation to breast cancer and to p53 mutations are being determined.

Figure 1



A population-based case-control study of breast cancer was conducted between 1986 to 1991; blood and tissue have been stored. There were 371 postmenopausal and 301 premenopausal women with breast cancer and 438 and 316 age-matched controls, respectively. A comment by a reviewer of our earlier report asked for a flow sheet showing the number of contacts, participants, etc. This is shown in Figure 1. One limitation of this study is that the actual number of blood donors was substantially less than the number of contacts. However, and importantly, our collaborators at the University of Buffalo examined the characteristics of those who gave blood and those who did not, versus characteristics of contacts. In premenopausal cases, there was no statistically significant difference in socioeconomic, hormonal,

reproductive or dietary risk factors. In postmenopausal cases, there were greater differences, but not statistically significantly different. Postmenopausal controls providing blood samples had a greater mean number of pregnancies (3.5 vs. 2.9; $P<0.01$) and fewer years of smoking (30 vs. 33; $P=0.14$). There were more never smokers among blood donors (67% vs. 62%; $P=0.07$).

This project planned to perform NAT2, GSTM1, CYP1A1, CYP2D6 genotyping for both premenopausal and postmenopausal cases, and examine these biomarkers in relation to smoking and diet. All assays have been completed, several publications have been produced (see below), additional manuscripts are submitted and other analyses are continuing. Additionally, genotyping for CYP2E1, APOE, aldehyde dehydrogenase, glutathione-*S*-transferase theta (GSTT), N-acetyltransferase 1, superoxide dismutase (SOD), and microsomal epoxide hydrolase (MEH) genetic assays have been completed. The decision to study these genes was made in the context of a priori hypotheses relating to gene-environment interactions. They all have polymorphisms which are associated with changes in carcinogen metabolic activation, detoxification or carcinogen-DNA adduct formation. These studies also have resulted in several publications (see below), additional ones are submitted and other analyses are ongoing.

We also proposed to determine the p53 mutational spectra to see if we can find associations with gene-environment interactions. The original plan was to p53 mutational spectra for informative cases identified by single stranded conformational polymorphism analysis and immunohistochemical staining, but we abandoned this strategy for simpler and more reliable microarray chip technology, using the Affymetrix p53 system. We validated this method by comparing a subset of samples that also were manually sequenced. There was difficulty in obtaining blocks from local hospitals, which necessitated a one-year no cost extension to the grant. To date, we have obtained 300 blocks, but an additional 150 blocks have been promised from Millard Hospital and Roswell Park Cancer Center. Thus this study is still ongoing (additional funding has been received from the NCI Intramural Program) so that it can be completed. Persons with mutations will be categorized by mutation and hypothesized chemical etiology will be compared to persons with other types of p53 mutations (four for each case) and also to controls without cancer (ten for each case). Odds ratios and logistic regression will address the association of genetic polymorphisms and exposures as a risk for p53 mutation and breast cancer, adjusting for other risk factors. We also will examine effect modification for other risk factors by genetic polymorphisms.

The originally proposed workscope was expanded to perform additional studies relating to findings in the first year of the award, specifically as they relate to smoking, smoking-related carcinogens and breast cancer. Thus, we began establishing human breast epithelial cell strains to examine the rate of carcinogen-DNA adduct formation from cigarette-smoke carcinogens, as well as the p53 and apoptosis response, with plans to determine interindividual variation. The purpose of these studies is to corroborate our epidemiological findings. Secondly, we have been developing a new carcinogen-DNA adduct procedure, nicknamed "CAP" for ^{14}C-Acetic Anhydride Postlabeling, which can be used in breast tissue analysis. These studies led to sufficient preliminary data to obtain a new DOD Breast Cancer Grant beginning this year. Separately, we embarked on a study to confirm our previous NAT2, smoking and breast cancer risk study (1) by examining this question in a case-series analysis from MD Anderson Cancer Center. This study will examine the question in different racial groups and also consider

survival. The study is not yet complete, but additional funding has been secured from the NIH Office of Minority Health, with a grant beginning in FY98. Finally, we examined nicotine addiction and genetic risk factors for addictive behaviors, in the context of a smoking cessation project, in order to identify risks for smoking addiction and smoking cessation strategies. This project also resulted in several publications (see below).

BODY

1. Collection of Tissue Samples and Tissue Preparation

- Tumor blocks for 300 cases have been obtained and sectioned, and the DNA has been extracted. This represents an additional 85 blocks from last year. We also know that 32 blocks have been inadvertently destroyed at a local hospital, and one cancer center refuses to provide the blocks to us. We expect to obtain an additional 150 blocks this year from Millard Hospital and Roswell Park Cancer Center. The former has agreed to provide the samples for a fee, which has been sent, and the latter is waiting only for IRB approval. In total then, we expect to analyze 450. This will represent the largest study to date of p53 mutational spectra and gene-environment interactions.

  Dr. Andrew Borkowski at the University of Maryland is provide a second histological review of breast cancer slides to confirm diagnoses and he will circle areas of tumor for microdissection (200 subjects completed to date).

- A mechanism for receiving fresh breast tissues from autopsy cases and reduction mammoplasties continues to go well. To date, we have established 52 strains from a total of 85 breast tissues received (not all strains are established from tissues collected), and culturing is now routine from both autopsy and surgical donors. Additionally, we have previously collected 150 frozen breast tissues from autopsy and surgical donors, many of the former who have also donated liver. All surgical cases have completed an epidemiological questionnaire. The established cell strains come from a subset of these tissue donors, so that we are establishing a resource where we can look at in vitro cellular responses and then examine the parent tissue for carcinogen adducts and metabolism, and also have epidemiological questionnaire data.

- DNA has been extracted from approximately 600 smokers and non-smokers enrolled in a study of tobacco addiction in collaboration with Georgetown University.

- Blocks are continuing to be received for a multiracial study of breast cancer in collaboration with MD Anderson Cancer Center. We sought a well-characterized study where we could replicate our earlier findings for NAT2, smoking and breast cancer (1), but also our primary goal is to examine gene-environment interactions for breast cancer risk and survival in a case-series analysis. Six hundred cases have been identified who were diagnosed from 1983 to 1993 and have had epidemiological questionnaires completed. These women include 400 Caucasians, 100 African Americans and 100 Hispanics. To date, 176 blocks have been received, but we continuing to receive blocks, and also to review medical records. In order to meet our accrual goals, we had increased the inclusion criteria from diagnosis before 1990 (from 1986), so as to continue to have more than 10 years of follow-up. We expect to have all blocks

collected this year, have DNA extracted and NAT2 genotyping completed. Additional funding for this project has come from a 3 year grant from the NIH Office of Minority Health.

2. Genetic Polymorphism analysis

- Our initial focus was to study tobacco smoking as a risk factor for breast cancer. While smoking is generally considered not to be a risk factor for breast cancer, based on numerous epidemiological studies, it was our hypothesis that smoking would indeed be a risk factor in some women, but not others. When studied together as a homogenous population, the risk would not be observable. Thus, to test this hypothesis, we studied risk in the *N*-acetyltansferase gene (*NAT2*), because this gene functions as a detoxification pathway for aromatic amines, for which there is ample experimental evidence to suggest that aromatic amines would be a human breast carcinogen. The *NAT2* genetic polymorphism, which predicts rapid or slow acetylation, was tested in 304 breast cancer cases and 327 community controls. Neither smoking or the *NAT2* gene by themselves were risk factors, but when the women were stratified by smoking risk based on acetylation status, in postmenopausal women, smoking carried a risk of up to 4.4 (95% C.I.=1.4, 10.8) in slow acetylators, which was consistent with several different types of analyses for this data set. There was no similar findings for premenopausal women. A manuscript was published summarizing these findings in the Journal of the American Medical Association in 1996. See Appendix B. There are now two publications which have not replicated our results (2,3), but we are aware of 2 unpublished studies which are consistent and two others that are not. Separately, data from the laboratory of David Phillips indicates that breast cells are capable of activating aromatic amines, which are higher in women who are slow acetylators.

  Subsequently, we also examined the NAT2 genotypes in relation to consumption of meats, as a surrogate for heterocyclic amine consumption. While our questionnaire is appropriate for meat consumption, it is known that cooking practices is what determines the actual quantity of heterocyclic amines. Thus, our estimates of risk are approximate. In our study, we did not find a risk related to meat consumption modified by NAT2. A manuscript describing these results is in press for the International Journal of Cancer. See Appendix C.

  Because of a previous study which suggested that NAT2 might interact with smoking to increase the risk of spontaneous abortions, we examined our data but did not find a similar risk. This data was published in Epidemiology. See Appendix D.

  NAT1 genotyping has been completed for postmenopausal women and premenopausal women (Appendix E). The genotypic frequency is similar to previous reports in the literature. Quality control analyses was completed and

the data did not increase a risk for the NAT1, either with or without smoking. See Appendix E. The current analysis examines allele numbers 3, 4, 10 and 11. But, while previous data has suggested the *10 allele was associated with increased activity and risk of colon and bladder cancer, subsequent data has indicated that *10 allele is actually not a functional polymorphism. Also since then, two additional low frequency alleles have been identified are functional, and we decided to examine these because they are likely more relevant. But, depending on the risk estimates, we might not have enough statistical power. These assays are now in progress. An abstract was published for the 1998 Annual meeting of the AACR summarizing the above results. A manuscript for submission is being prepared.

- A commonly accepted risk factor for breast cancer is alcohol consumption, and the findings are more frequently reported in premenopausal rather than postmenopausal women. It is currently unknown what might be the carcinogenic agents in alcoholic beverages. One candidate is ethanol, because ethanol is oxidized to acetaldehyde, which is mutagenic and carcinogenic in laboratory animals. The principle pathway for ethanol oxidation is through alcohol dehydrogenase. In order to study the risk of alcohol drinking in the context of ethanol metabolism, we studied the alcohol dehydrogenase 3 gene (*ADH3*). In this study, we found that women who would be predicted to have an increased capacity to form acetaldehyde ($ADH3^{1-1}$), had an odds ratio of 3.0 (95% C.I.=1.3, 6.6) in high drinkers compared to low or nondrinkers. Compared to women who would have a decreased capacity ($ADH3^{2-2}$), there was a 3.3-fold risk (95% C.I.=0.9, 12.9). This work has been accepted for publication in Cancer Causes and Control, and is included in Appendix F.

- Apolipoprotein E is involved in the production of VLDL and other parts of cholesterol metabolism. Several studies have related low cholesterol levels to breast cancer risk. The apoE gene is polymorphic, where some variants raise cholesterol levels and others lower them. We therefore measured apoE genotypes in both the pre- and postmenopausal women. The analysis did not indicate a main effect risk of the gene on breast cancer risk. When considered in the context of serum cholesterol and triglycerides, there was an increased risk for women with the highest tertile of triglycerides. This work has been accepted for publication in Molecular Carcinogenesis. See Appendix G.

- Our previous results suggested that a polymorphism in cytochrome P450IAI is related to breast cancer in postmenopausal women with low tobacco use. There also was a non-significant trend for GSTM1 in younger postmenopausal women. Both of the enzymes are involved in the activation and detoxification, respectively, of polycyclic aromatic hydrocarbons. We have completed the analysis for these genes for premenopausal women, and also for GST-theta for all women. Preliminary analyses did not indicate an increase risk. (Genotype frequencies are listed in Appendix H). Further analysis is ongoing and we will

submit a manuscript for publication. We also have examined the postmenopausal data for *CYP1A1* in relation to polychlorinated biphenyl (PCBs) body burdens. It was previously published that PCBs might be related to breast cancer risk, although subsequent data, including from this study did not show this. But, we hypothesized that one way that PCBs might contribute to breast cancer risk was through the induction of cytochrome P450s and attendant increased metabolic activation. Our data indicated that the *CYP1A1* genetic polymorphism was a risk factor in breast cancer in women with PCB levels above the median. This work has been published in Cancer, Epidemiology, Biomarkers and Prevention. See Appendix I.

- Another enzyme involved in this pathway is microsomal epoxide hydrolase. There are two polymorphic sites that result in a decrease of activity by 40%. The measurement of MEH in pre-and postmenopausal women is complete. This gene was not found to be a risk factor for breast cancer. A manuscript is currently being prepared. See Appendix J for genotype frequencies.

- Cytochrome P450IID6 has been associated with lung cancer and breast cancer. Its metabolic substrate is unknown, but it may be a tobacco-specific nitrosamine. All genotyping for 3 different polymorphic sites is completed. Statistical analysis is beginning, and a manuscript will be submitted. Genotype frequencies are indicated in Appendix K.

- There is a genetic polymorphism in manganese superoxide dismutase (SOD2), due to a valine to alanine substitution in the protein, that may block the transport of the enzyme into the mitochondrion. We hypothesized that the SOD2 A allele could result in a protein of decreased efficacy in fighting oxidative stress, and thus lead to an increased risk of breast cancer. We were also interested in whether in a diet rich in sources of antioxidants could ameliorate breast cancer risk associated with the SOD2 A allele. Oxidative stress, resulting from the imbalance between prooxidant and antioxidant states, damages DNA, proteins, cell membranes and mitochondria. There is evidence for a role of oxidative stress in human breast carcinogenesis. Dietary sources of antioxidants (chemical) and endogenous antioxidants (enzymatic), including superoxide dismutase, can act to reduce the load of oxidative stress. Using a restriction fragment length polymorphism for SOD2 that distinguishes the A and V alleles, we characterized SOD2 genotypes in relation to breast cancer risk and also evaluated the effect of the polymorphism on risk among low and high consumers of fruits and vegetables, as well as specific dietary and supplemental sources of antioxidants. Premenopausal women who were homozygous for the A allele had a four-fold increase in breast cancer risk in comparison to those with V alleles (odds ratio [OR]=4.3, 95% confidence interval [CI], 1.7-10.8). Risk was most pronounced among women below the median consumption of fruits and vegetables, and of dietary sources of ascorbic acid and a-tocopherol, with little increased risk for those with diets rich in these foods. Relationships

were weaker among postmenopausal women, although the SOD2 AA genotype was associated with an almost two-fold increase in risk (OR=1.8; CI, 0.9-3.6). No appreciable modification of risk by diet was detected for these older women. This work was published in Cancer Research (See Appendix L).

3. P53 Mutational Spectra Analysis

- Immunohistochemistry staining: Blocks from 300 individuals have been obtained and have been sectioned. P53 immunohistochemistry staining has been done for 329 samples and the rest are in progress. The staining results are as follows:

Score	N
0	200
1	28
2	24
3	77

- The p53 mutational spectra is currently being determined using the Affymetrix p53 chip. We first needed to validate the method and determine the cut off for identifying mutations. We did this using cell lines and previously sequenced set of lung cancer samples. We subsequently performed direct sequencing for 68 Buffalo breast cancer cases and compared the results using the chip technology. We found that a 96% agreement among all samples (the two samples that were discordant are being resequenced). Interestingly, the chip was more reliable because it was able to call equivicable direct sequencing results. It also is substantially easier and quicker. We determined a cut-off score of 15, by sequencing all exons that provided scores of 13 or higher. These results are summarized in Appendix M. A total of 85 samples have been analyzed to date by the chip technology. 25 samples had detectable p53 mutations and 60 had wildtype sequences for exons 2 - 11. Additional samples will be analyzed using already secured Intramural Research Program funding.

4. Ancillary Studies

- *Breast Cell Strain Studies.*

We have developed the technique in our laboratory, based upon previously published methods, to isolate breast epithelial cells and culture them in a sterile environment. Thus far we have established 53 cell strains. In these cells, we have determined that 4-aminobiphenyl is metabolically activated through cytotoxicity experiments, and have determined optimal timing and dose response relationships. Both metabolites of 4-ABP and parent 4-ABP are active in producing cell death, suggesting the presence of NAT1 and CYP1A2 in these cells. We are also now identifying the p53 induction in relation

to the exposures, but have identified problems in reliable fixation without affecting P53 status. We are now quantitating p53 response using Western Blot. The current data is presented in Appendix N.

Verification of adduct formation in breast epithelial cells: To verify that adducts are actually formed by the mammary epithelial cells in vitro, calf thymus DNA was treated with N-OH-ABP in solution for 24 hrs. 4-ABP adducts were detected by HPLC when this DNA was digested with NP1, alkaline phosphatase, and snake venom phosphodiesterase

A strain of mammary epithelial cells (3 T75 flasks each) was treated with either 4-ABP or N-HO-ABP resulting in 300 - 500 ug total DNA. The DNA was digested with NP1, alkaline phosphatase, and snake venom phosphodiesterase and the resulting nucleosides and adducts underwent HPLC purification but did not give a peak at 30 min. corresponding to 4-ABP-dG standard.

Similarly, mammary cells were treated with dichlorobenzene-ABP (a very reactive synthetic intermediate 4-ABP). Cytotoxicity assays were performed and the cells showed dose related death, indicating adduct formation. However, adducts were not detected after enzymatic digestion and analysis by HPLC.

Cell strain (LHC 10340) was treated in 3 T-75 flasks each at either 0, 3 or 300 uM doses of 14C-4-ABP for 1 hour. The DNA was extracted, quantitated and counted by scintillation counting. No counts were found above background, as expected. The DNA was sent to Lawrence Livermore National Labs (LLNL) for Atomic mass spectrometry analysis. Results from LLNL indeed show a 100 fold increase in 14C level within the treated cells. The were detected adducts are 243000/10^12 nucleotides at the 3 uM dose or 18500000/10^12 at the 300 uM dose. Untreated controls had background levels of 14C.

Replication of the experiment with the same cells and same doses also showed a 100 fold increase in the 14C levels in the 300uM dose over the 3 uM dose.

Quantitation of p53 induction in human breast epithelial cells after treatment with N-OH-DNA and 4-ABP: Determination of Western blotting conditions: A single 35 mm plate of mammary cells was determined to give enough protein for several western blots (about 100 ug total protein of which 20 ug is loaded per well). The procedure for the blotting is: 1. Cells are grown in 35 mm plates until ~60% confluent (about 1 week) in MEGM. The media is removed and fresh media with 30 uM 4-ABP or N-OH-ABP is added (in 2 ml of media) for various times to generate a time course for appropriate treatment. The cells are harvested by lysing and scraping the dishes with cell scrapers. The lysate is then quantitated by Pierce BCA protein assay. Protein lysates (20 ug) are loaded on a pre-cast 10% Tris-glycine polyacrylamide gel and eletrophoresed at 200V until the marker dye is at the bottom of the gel (~1 hr). The protein is transferred to a nitrocellulose filter by standard electrophoretic transfer. Bands (p53) are visualized by

chemiluminescence detection on film after incubation with anti-p53 (Ab-6; Calbiochem) and goat-anti-mouse horse radish peroxidase (Amersham). A lane of Beas-2B cell lysate which is known to overexpress p53 is to used on each gel to control for gel-to-gel variability and serve as a positive control for p53. B-actin expression by western blotting is used as the internal control for protein loading.

Repeatedly, endogenous p53 levels are very high, even in untreated cells. This is not apparently due to cell confluency as even sparse cells populations give high p53 levels. Although B-actin blots are clean and informative, when protein levels are used to correct for lane loading, quantitation becomes more and more speculative. Because the endogenous p53 is so high, it is also difficult to see and real differences in the levels of p53.

In addition, reproducibility of p53 induction and its detection are a concern. At a single 12 hr time point after treatment of primary breast epithelial cells with 30 uM 4-ABP, three identical dishes were lysed and the lysate run on as western blot. The recovery is 1125 +/- 322 densitometry units which is 30% variability. Ideally, 15 % variability would be desirable to be confident in your reproducibility and any fold induction. These concerns about the insensitivity of the western blot have forced us to reconsider our method for quantitating p53 protein and we are now exploring a quantitative ELISA format for p53 quantitation.

- *Carcinogen DNA Adduct Studies.*

We have been developing the CAP (^{14}C-acetylation procedure) for the detection of DNA adducts based upon micropreparative techniques, ^{14}C-acetic anhydride acylation postlabeling, and quantitation by accelerator mass spectrometry (AMS) (4). This work has recently been funded through the peer review Department of Defense breast cancer program for application in breast tissues. There are several important advantages for this methodology over previous adduct detection methods overall, and for ^{32}P-postlabeling in particular. This method is highly chemically specific so that we can be sure that we are measuring what we want to measure. This happens because of two chromatographic methods, namely immunoaffinity chromatography (IAC) and high performance liquid chromatography (HPLC). Second, the labeling method is more reliable because it uses a predictable chemical reaction. Third, we retain the same or a better degree of sensitivity because of AMS, which is an ultrasensitive ^{14}C detection unit used for ^{14}C dating archeological samples and biophysics applications. Under the direction of our collaborator, Dr. Kenneth Turteltaub, AMS has a documented sensitivity of 10^{-20} moles of ^{14}C (5). This leads to a theoretical detection limit of 1 adduct in 10^{-13} nucleotides (!) (6), and represents a 1000-fold improvement over other adduct methodologies. Our current real limit of detection is 300 attomol (10^{-18} mol). It will allow us to use only micrograms or less of human DNA for analysis. We have been using this method for the adduct systems that we know best, specifically BP-dG

and 4ABP-dG. The scheme and methodology are described in Appendix O.

- *Tobacco Addiction Studies*

An understanding of why people smoke cigarettes can have an important impact upon smoking prevention and cessation. People smoke cigarettes to maintain nicotine levels in the body, and nicotine has been implicated in the stimulation of brain reward mechanisms via central neuronal dopaminergic pathways. We recruited smokers (n=283) and nonsmokers (n=192) through local media for a case-control study of smoking. Following informed consent and a behavioral questionnaire, smokers underwent a single minimal contact session of smoking cessation counseling, and then were followed for up to one year. Thus far, we have found that there is an interaction for polymorphisms with the dopamine transporter gene and the dopamine D2 receptor for smoking risk (P=0.001) and the combination of the two genotypes reduces the risk of smoking by more than half. This manuscript has been published in Health Psychology. See Appendix P.

In this study, we also evaluated the association of smoking and smoking cessation with a dopamine D4 receptor 48 base pair variable nucleotide tandem repeat polymorphism, where the 7 repeat allele (D4.7) reduces dopamine affinity. The frequency of the dopamine D4 receptor genetic polymorphism using PCR was determined and individuals were classified by the number of repeat alleles (2-5 repeats as "S" and 6-8 repeats as "L"). Persons with those genotypes including only S alleles (homozygote S/S) were compared with those with at least one L allele (heterozygote S/L and homozygote L/L). The data showed that the S allele interacted with depression to increase the risk of smoking. A manuscript was published by Health Psychology (See Appendix Q). We also found that the L allele increased smoking risk in African Americans. This paper has been accepted by Cancer, Epidemiology, Biomarkers and Prevention (See Appendix R). We also have studied genetic polymorphisms in the tyrosine hydrolase and serotonin transporter genes, which did not yield positive associations. These manuscripts have been published in Pharmacogenetics and Cancer, Epidemiology, Biomarkers and Prevention (see Appendix S and T).

CONCLUSIONS

The findings of an association of smoking and breast cancer in Caucasian women with the slow NAT2 acetylation genotype is very important because approximately 50% of women are slow acetylators. This results in a large attributable risk. The findings need to be reproduced and examined in other races. Such a study by us is underway in collaboration with the MD Anderson Cancer Center. Data from other studies are conflicting but there also are different methodological designs. Laboratory studies also need to corroborate this finding by examining the metabolic potential in rapid and slow acetylators. Recent studies showing that breast cells contain acetyltansferase activity and our studies described above are consistent with the epidemiological data, but adduct studies also are needed. The development of the acetic

anhydride postlabeling procedure will provide data for intermediate endpoints. Thus, the application of this procedure for aromatic amine adducts in cell strains and parent tissues may provide important corroborative data for the epidemiological findings. Finally, the p53 mutational spectra will also provide data on intermediate endpoints and also possibly identify the effects of acetyltansferase on ultimate outcome. While it has been difficult to obtain blocks in the past, we now have in hand a significant number of samples, more will come and we expect that this mutational data on such a large number of subjects will be very important. As follow-up to smoking related risk, the ability to prevent smoking addiction and increase smoking cessation has the greatest potential impact from a public health and individual health perspective. The identification of polymorphisms in the dopamine receptor genes and dopamine transporter genes may be able to identify optimal prevention strategies.

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LIST OF PERSONNEL
Radoslav Goldman, Ph.D.
Amy Warren, Ph.D. (Terminated in 1997)
Cong Lai, Ph.D.
Bryan Cobb (Terminated in 1997)
Shiva Krishnan

(Note that other persons are actively working on this project and are supported by NIH intramural funds, as described in the original proposal.)

APPENDIX A – Letter describing Revised Workscope

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

August 21, 1995

Juanita Bourne
U.S. Army Medical Research Acquisition
Attn: MCMR-RMA-RG
Fort Detrick
Frederick, MD 21702, MD 21702

Re: DAMD17-95-1-5022 -- "Environmental Exposures ..."

Dear Ms. Bourne:

Per our discussion on August 16, 1995, we are requesting a budget modification and permission to expand the work scope for our above cited grant. This request is being made because we have found some very interesting results that should be pursued with expanded studies. Secondly, and as you are aware, we have encountered some difficulties in applying our 30% indirect costs because NIH has no mechanism for billing such under an Interagency Agreement. Therefore, we are proposing to modify the budget and redirect the indirect costs to direct costs, except for 8.3% of the 30% that actually can be billed as part of the overhead on salaries. I have attached a revised budget.

We are proposing to expand our work scope by performing additional studies on the role of genetic polymorphisms in breast cancer. Under the existing grant, we have found that women who are slow acetylators of the NAT2 gene and are cigarette smokers have an increased risk of breast cancer. This is a novel finding in that cigarette use is not commonly considered a breast cancer risk factor. Separately, our data also suggests that persons with variants in the cytochrome P4501A1 and glutathione-*S*-transferase M1 genes might be at increased breast cancer risk if they are lighter cigarette smokers or younger postmenopausal women, respectively. The proposed expansion of the work scope, redirecting the indirect costs, will allow us to perform corroborative studies of these findings. Specifically, we will look at other populations for similar findings, attempting to replicate our first findings. We will perform laboratory studies of metabolism in breast tissues to confirm that breast tissues have the metabolic machinery to form DNA adducts. We also will examine risk in the context of smoking addiction and genetic polymorphisms, identifying polymorphisms that place people at

greater risk for addiction. These new polymorphisms can then be examined in our breast cancer case-control study so see if there is an added risk, or we can define new subpopulations, that is based upon addiction rather than simply number of cigarettes consumed. This expansion will involve collaborations with Dr. Margaret Spitz and Melissa Bondy at M.D. Anderson in Houston, Texas and Dr. Carynn Lerhman at Georgetown, University (Washington, D.C.).

The budget modification reflects the following:

1. Increase expense for DNA extractions to allow us to study additional populations.

2. Increase expense for genetic polymorphism analysis to allow us to study additional populations.

3. Increase travel expenses to allow all three investigators to attend national meetings and support additional travel to collaborators.

4. Add new line item for ancillary studies that will allow us to study metabolism and DNA adduct formation in breast tissues.

5. Add line item for equipment that allow us to purchase DNA thermocycler that supports 96 well applications and an automated ELISA reader. This equipment will allow us to increase our capacity for genotyping and significantly reduce the cost. Thus, we will be able to perform the expanded work without increasing labor costs.

6. Our cost for performing histology sections has decreased so that I have decreased this line item and increased the line item for genotyping to allow for the expanded work scope.

I hope this expansion will be acceptable to you. Of course, all proposed activities in the original proposal will be completed and the total appropriation of $486,665 over three years will not be exceeded.

Thank you for your consideration in this matter. If you need further details, please let me know. I look forward to your response.

Sincerely,

Peter G. Shields, M.D.
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Molecular Epidemiology Section
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cc: Dr. C. Harris
Bonnie Richards
Betty Fitzpatrick
Jenny Kiesewetter
Dr. J. Rice

APPENDIX B – N-Acetyltransferase (NAT2), Cigarette smoking and breast cancer risk

Ambrosone, C. B., Freudenheim, J. L., Marshall, J. R., Graham, S., Vena, J. E., Brasure, J. R., Michalek, A. M., Bowman, E. D., Gillenwater, K., Harrington, A. M. and Shields, P. G.: N-Acetyltransferase (NAT2), Cigarette smoking and breast cancer risk. J. Am. Med. Assoc., 276: 1494-1501, 1996.

Preliminary Communication

Cigarette Smoking, *N*-Acetyltransferase 2 Genetic Polymorphisms, and Breast Cancer Risk

Christine B. Ambrosone, PhD; Jo L. Freudenheim, PhD; Saxon Graham, PhD; James R. Marshall, PhD; John E. Vena, PhD; John R. Brasure; Arthur M. Michalek, PhD; Rosemary Laughlin, PhD; Takuma Nemoto, MD; Kari A. Gillenwater; Anita M. Harrington; Peter G. Shields, MD

Objective.—To determine if *N*-acetyltransferase 2 (*NAT2*) polymorphisms result in decreased capacity to detoxify carcinogenic aromatic amines in cigarette smoke, thus making some women who smoke more susceptible to breast cancer.

Design.—Case-control study with genetic analyses. DNA analyses were performed for 3 polymorphisms accounting for 90% to 95% of the slow acetylation phenotype among whites.

Setting and Participants.—White women with incident primary breast cancer (n=304) and community controls (n=327).

Results.—Neither smoking nor NAT2 status was independently associated with breast cancer risk. There were no clear patterns of increased risk associated with smoking by NAT2 status among premenopausal women. In postmenopausal women, NAT2 strongly modified the association of smoking with risk. For slow acetylators, current smoking and smoking in the distant past increased breast cancer risk in a dose-dependent manner (odds ratios [95% confidence intervals] for the highest quartile of cigarettes smoked 2 and 20 years previously, 4.4 [1.3-14.8] and 3.9 [1.4-10.8], respectively). Among rapid acetylators, smoking was not associated with increased breast cancer risk.

Conclusions.—Our results suggest that smoking may be an important risk factor for breast cancer among postmenopausal women who are slow acetylators, demonstrate heterogeneity in response to carcinogenic exposures, and may explain previous inconsistent findings for cigarette smoking as a breast cancer risk factor.

JAMA. 1996;276:1494-1501

CIGARETTE SMOKING is a risk factor for many human cancers, at organ sites with both direct and indirect contact with tobacco smoke.[1] Most epidemiologic studies have not found a clear association between smoking and breast cancer risk[2-12]; some report elevated breast cancer risk,[13-21] while others report decreased risk.[22-24] No study has considered genetic variability in susceptibility to cigarette smoke carcinogens.

For editorial comment see p 1511.

Mutagens from cigarette smoke come into direct contact with breast epithelial cells. Nipple fluid aspirated from smokers contains nicotine metabolites[25] and is mutagenic.[26] Tobacco-related carcinogen–DNA adducts in human breast tissue have been identified.[27,28] Aromatic amines found in tobacco smoke, such as 4-aminobiphenyl and β-naphthylamine, could be mutagenic and carcinogenic because they are metabolically activated and cause DNA damage in human breast epithelial cells,[29,30] transform cultured mouse mammary epithelial cells,[31] and induce mammary tumors in laboratory animals.[32,33]

Aromatic amines are detoxified and/or bioactivated by xenobiotic metabolizing enzymes, including *N*-acetyltransferase 2 (NAT2). The activity level of this enzyme determines the rates of detoxification and activation of aromatic amines in

From the National Center for Toxicological Research, Division of Molecular Epidemiology, Jefferson, Ark (Dr Ambrosone); Departments of Social and Preventive Medicine (Drs Ambrosone, Freudenheim, Graham, Marshall, Vena, Michalek, and Laughlin and Mr Brasure) and Surgery (Dr Nemoto), State University of New York at Buffalo; and Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, Md (Mss Gillenwater and Harrington and Dr Shields).

This work was a collaborative effort by the Department of Social and Preventive Medicine, State University of New York at Buffalo, and the Laboratory of Human Carcinogenesis, National Cancer Institute. It is solely the responsibility of the authors and does not necessarily represent the views of the National Cancer Institute.

Reprints: Christine B. Ambrosone, PhD, National Center for Toxicological Research, Division of Molecular Epidemiology, 3900 NCTR Rd, Jefferson, AR 72079 (e-mail: cambrosone@nctr.fda.gov); or Peter G. Shields, MD, Bldg 37, Room 2C16, Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, MD 20892 (e-mail: peter_g_shields@nih.gov).

humans.[34] For NAT2, phenotypic and genotypic assays are used to classify individuals as rapid or slow acetylators.[35-38] Slow NAT2 acetylators are at increased risk for urinary bladder cancer, particularly with occupational exposure to aromatic amines.[39,40] NAT2 is also involved in *O*-acetylation of amine metabolites, and rapid NAT2 acetylation of heterocyclic amines formed in cooking meat may be related to colon cancer risk.[41-43] Examination of the NAT2 phenotype in breast cancer has shown inconsistent results,[44-48] but phenotypes may be altered by disease or treatment status. We hypothesized that polymorphisms in NAT2 may result in decreased capacity to detoxify carcinogenic aromatic amines in cigarette smoke, thus increasing susceptibility to breast cancer.

SUBJECTS AND METHODS

Study Population

These analyses are based on data from an earlier case-control study (1986-1991) of 617 premenopausal and 933 postmenopausal white women in New York.[49] Protocols for the initial study and the nested study of genetic polymorphisms were reviewed by the State University of New York at Buffalo Institutional Review Board. Written informed consent for interview and medical record review was obtained from participants. The criteria for postmenopausal status in women under the age of 50 years were natural menopause, bilateral oophorectomy, or irradiation to the ovaries; in women aged 50 years and older, the criterion was cessation of menstruation.

Cases, identified from all major hospitals in Erie and Niagara counties, were women with incident primary, histologically confirmed breast cancer. Controls were frequency-matched to cases by age and county of residence. Women under 65 years of age were randomly selected from the New York State Motor Vehicle Registry, while those 65 and over were selected from Health Care Financing Administration rolls. Of premenopausal women contacted, 66% of eligible cases (n=301) and 62% of eligible controls (n=316) participated, and of postmenopausal women, 54% of cases (n=439) and 44% of controls (n=494) participated. After informed consent was obtained, an in-person interview was administered to assess medical, reproductive, and lifetime smoking history and usual food consumption 2 years prior to the interview. While efforts were made for interviewers to be blinded to case-control status, in many cases, due to the nature of the interview, this was not possible. However, during the interview, interviewers and investigators were not aware of the genetic hypotheses.

About 45% of premenopausal and 63% of postmenopausal women interviewed provided blood samples, with written informed consent for blood analyses. In premenopausal women, there were no statistically significant ($P<.05$) differences in socioeconomic, hormonal, reproductive, or dietary factors between those giving and not giving blood, and in postmenopausal cases, only slight differences. Postmenopausal controls providing samples had a greater mean number of pregnancies (3.5 vs 2.9, $P<.01$) and fewer years of smoking (30 vs 33, $P=.14$) than those who did not. There were more never-smokers among controls who consented to phlebotomy (67% vs 62%) ($P=.07$). DNA analyses were performed for cases and controls whose specimens had adequate DNA.

Laboratory Methods

Whole blood was collected in plain red-top tubes and transported within 3 hours, on ice, to the laboratory, where it was processed immediately. Specimens were centrifuged and serum was pipetted into vials. Two aliquots of clots (1 mL each) were preserved and stored at −70°C. For this study the 1-mL blood clots were thawed, mechanically disrupted (Brinkman Instruments Polytron, Westbury, NY) for 10 seconds, and digested with proteinase K (Life Technologies, Grand Island, NY) at 55°C for 8 to 12 hours in 5 mL of buffer containing 10-mmol/L Tris hydrochloride, pH 7.8; 50-mmol/L ethylenediaminetetraacetic acid (EDTA); and 0.5% sodium dodecyl sulfate. Phenol (5 mL) was added to the sample, which was mixed gently for 1 hour. After centrifugation, the aqueous phase was transferred to a clean tube. The phenol phase was mixed with additional buffer (2 mL of 10-mmol/L Tris hydrochloride, pH 8.0, and 0.1-mmol/L EDTA) and centrifuged, and the aqueous phases were combined. The sample was extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1 vol/vol), and DNA was precipitated with sodium acetate (pH 5.2; final concentration, 0.3 mol/L) and 2.5 volumes of ethanol. Yields of DNA were 5 to 20 μg per sample. Genomic DNA (30 ng) was amplified by polymerase chain reaction (PCR) in the presence of primers specific for *NAT2*[36] (5'-TCTAGCATGAATCACTCTGC and 5'-GGAACAAATTGGACTTGG), buffer (10-mmol/L Tris hydrochloride, pH 8.3; 50-mmol/L potassium chloride; and 3-mmol/L magnesium chloride), 0.15-mmol/L 2'-deoxynucleoside-3'-triphosphates (Pharmacia, Piscataway, NJ); and 2.5 U of *Taq* polymerase (Perkin Elmer, Norwalk, Conn) in a total volume of 100 μL. An aliquot (18 μL) was then subjected to restriction fragment length polymorphism (RFLP) analysis for the $C^{481}T$ (*Kpn*I) (New England Biolabs, Beverly, Mass), $G^{590}A$ (α*Taq*I) (New England Biolabs), and the $G^{857}A$ (*BamH*I) (New England Biolabs) polymorphisms, according to the manufacturer's instructions (nomenclature for *N*-acetyltransferases as published[50]). These 3 polymorphic sites predict 90% to 95% of slow acetylation phenotype among whites.[36,38] Agarose gel electrophoresis (2.2% for the $C^{481}T$ and $C^{857}A$; 4% for the $C^{590}A$; NuSieve: Agarose, 3:1, FMC Bioproducts, Rockland, Me) was used to detect RFLP patterns. Each individual was classified as a rapid acetylator (carrying 0 or 1 slow acetylator mutation) or slow acetylator (carrying 2 slow acetylator mutations).[36,37] Assays were performed and interpreted by 2 of the authors (A.H. and P.G.S.), who were blinded to subject status. A second analysis was done to confirm the original findings. In the second analysis, 33 samples were excluded because of faint electrophoretic bands due to suboptimal DNA quality.

Statistical Analyses

Bivariate analyses were used to examine the association between NAT2 status and breast cancer risk. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression and adjusted for potential confounding factors including age, education, body mass index (weight in kilograms divided by the square of height in meters with weight being the reported value 2 years prior to interview), age at menarche, age at first pregnancy, reported family history of breast cancer (mother and/or sister), and age at menopause. Hormone replacement therapy was not associated with breast cancer risk, and its inclusion in the model did not affect risk estimates. Unadjusted and adjusted ORs were similar; adjusted ORs are shown. The *P* for trend was calculated as level of significance of the β coefficient (indicative of the amount of increase in risk per unit change in the independent variable) for each continuous variable in the logistic regression model with relevant adjusting variables.

Smoking effect in relation to breast cancer risk was examined within strata of NAT2 genotypes. Association between risk and recent smoking (cigarettes smoked 2 years previously) and smoking in the distant past (cigarettes smoked 20 years previously) was assessed. Past smoking was assessed due to probable latency for breast carcinogenesis and possible damage to breast epithelial cells during breast growth. Packs per average year was calculated as weighted average of daily use, and

total duration of smoking was assessed. Pack-years, an estimate of total smoking exposure, was calculated as weighted average of daily use multiplied by total years of smoking. We had smoking information of 2, 10 (data not shown), and 20 years before the interview. The weighted average was the number of cigarettes smoked at each time period multiplied by 6 years and divided by the total number of years of smoking exposure. Smoking-related variables were stratified into quartiles based on approximately uniform distribution of smoking in controls, with never-smokers as referent category. When we assessed smoking 2 and 20 years previously, we excluded persons not smoking then but having previously smoked. Thus, the sample size varied among smoking variables.

Postmenopausal controls consenting to phlebotomy smoked less than nonparticipating controls. To eliminate this nondifferential bias and supplement assessment of acetylator status effects, 2 additional approaches were used. One was a case-series design in which smoking-related variables were regressed on acetylator status among cases only, comparing genotypes in terms of the degree to which smoking was a risk factor. The resultant OR was the ratio of risk associated with smoking for slow vs rapid acetylators, a supported method for studies incorporating genetic markers. Analyses based only on cases may offer better precision for estimating gene-environment interactions than those based on case-control data.[51,52]

The other approach was a model in which a subset of cases and subset of controls were matched on smoking by randomly selecting equal numbers of cases and controls within smoking quartiles (before stratification by genotype) with intent to eliminate selection bias related to smoking. Odds ratios were set to 1.0 at each quartile of exposure for smoking-related variables. Data were stratified by acetylator status, and associations between smoking and risk were evaluated.

RESULTS

Prevalence of genotypes as determined by assessment of each of the 3 *NAT2* mutant alleles (ie, $C^{481}T$, $G^{590}A$, and $G^{857}A$) among cases and controls is shown for premenopausal and postmenopausal women (Table 1). Interpretable PCR assays resulted in genetic data for 233 premenopausal and 398 postmenopausal women (83% and 71%, respectively) (Figure 1). There were no statistically significant differences between persons with and without successful PCR amplification for demographic, dietary, smoking, or reproductive variables (data not shown). Fifty-seven percent of premenopausal and 55% of postmenopausal women were classified as slow acetylators (Table 2). Slow acetylation status did not increase breast cancer risk.

The Student *t* test was used to assess statistical mean differences between rapid and slow acetylators in cases and controls for selected demographic, reproductive, and smoking-related variables (Tables 3 and 4). In premenopausal women, no statistically significant differences between these variables were seen for cases or controls, and in postmenopausal women, there were no statistically significant differences between rapid and slow acetylators for demographic or reproductive variables (Table 3). In postmenopausal controls, no statistically significant differences by genotype for smoking variables were seen, but cases differed in smoking histories by NAT2 genotype (Table 4): Slow acetylators smoked more at all times, suggesting a relationship to disease status among postmenopausal women with the slow acetylation genotype.

Among postmenopausal women, cigarette smoking and NAT2 genotype interacted with breast cancer risk. A statistical test for interaction that regressed NAT2 status, packs of cigarettes smoked per year, and their product on risk revealed that neither acetylator status ($P=.71$) nor smoking status ($P=.80$) was independently associated with breast cancer risk, but an interaction between acetylator status and smoking status contributed to postmenopausal breast cancer risk ($P=.05$).

After stratifying by NAT2 genotype, we evaluated associations between

Table 1.—*N*-Acetyltransferase 2 Polymorphisms for Cases and Controls: Western New York Breast Cancer Study, 1986 to 1991*

	Premenopausal			Postmenopausal		
	Case	Control	Total	Case	Control	Total
Rapid acetylators						
WT/WT	7	9	**16**	13	17	**30**
WT/$C^{481}T$	26	22	**48**	40	45	**85**
WT/$G^{590}A$	17	18	**35**	26	29	**55**
WT/$G^{857}A$	1	0	**1**	1	9	**10**
All	**51** (43)	**49** (43)	**100** (43)	**80** (43)	**100** (47)	**180** (45)
Slow acetylators						
$C^{481}T/C^{481}T$	26	24	**50**	42	49	**91**
$C^{481}T/G^{590}A$	27	26	**53**	43	48	**91**
$C^{481}T/G^{857}A$	4	2	**6**	5	4	**9**
$G^{590}A/G^{590}A$	9	13	**22**	13	8	**21**
$G^{590}A/G^{857}A$	2	0	**2**	2	4	**6**
$G^{857}A/G^{857}A$	0	0	**0**	0	0	**0**
All	**68** (57)	**65** (57)	**133** (57)	**105** (57)	**113** (53)	**218** (55)

*WT indicates wild-type allele in which the $C^{481}T$, $G^{590}A$, and $G^{857}A$ slow acetylator mutations are absent. Slow acetylators carry 2 of any of these mutations. All others are classified as rapid acetylators. Values in parentheses represent percentages of rapid and slow acetylators.

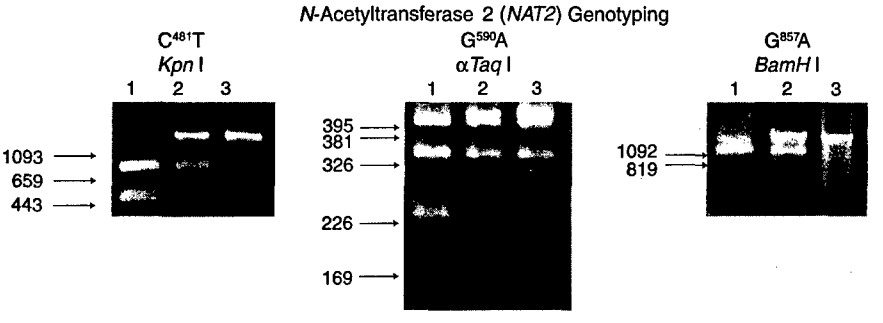

Figure 1.—Detection of NAT2 slow acetylator mutations in polymerase chain reaction (PCR) products. Genomic DNA was amplified by PCR[36] and digested by restriction enzymes. Slow acetylator mutations were examined: $C^{481}T$, by use of *Kpn* I, yields 659- and 443-bp (base pair) bands for wild-type alleles and a single 1093-bp band for the mutant alleles (left); $G^{590}A$, by use of α*Taq*I, yields 381-, 326-, 226-, and 169-bp bands for wild-type alleles and 395-, 381-, and 326-bp bands for the mutant alleles (center); and $G^{857}A$, by use of *BamH*I, yields 819- and 283-bp bands for the wild-type alleles (283-bp band not shown) and a single 1092-bp band for the mutant alleles (right). Thus, for each polymorphic site, homozygous wild-type (lane 1), heterozygote (lane 2), or homozygous mutant (lane 3) patterns are shown. The presence of 2 mutant alleles predicts the phenotypic slow acetylators.

Table 2.—*N*-Acetyltransferase 2 (*NAT2*) Polymorphisms and Breast Cancer Risk: Western New York Breast Cancer Study, 1986 to 1991*

NAT2 Genotype	Cases, No. (%)	Controls, No. (%)	Total, No. (%)	OR (95% CI)
		Premenopausal		
Rapid acetylators	51 (43)	49 (43)	100 (43)	1.0 . . .
Slow acetylators	68 (57)	65 (57)	133 (57)	0.9 (0.7-2.0)
		Postmenopausal		
Rapid acetylators	80 (43)	100 (47)	180 (45)	1.0 . . .
Slow acetylators	105 (57)	113 (53)	218 (55)	1.3 (0.8-1.9)

*The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated with unconditional logistic regression, with rapid acetylators (individuals with <2 mutant alleles) as the reference category (ellipses), adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer, and age at menopause for postmenopausal women.

Table 3.—*N*-Acetyltransferase 2 Polymorphisms and Demographic and Reproductive Characteristics for Cases and Controls: Western New York Breast Cancer Study, 1986 to 1991*

	Premenopausal			
	Cases		Controls	
Characteristic	Rapid Acetylators (n=51)	Slow Acetylators (n=68)	Rapid Acetylators (n=49)	Slow Acetylators (n=65)
Age, y	47 (4)	46 (3)	47 (3)	47 (4)
Education, y	14 (3)	14 (3)	13 (3)	14 (3)
Age at menarche, y	13 (1)	13 (2)	13 (2)	13 (2)
Age at first pregnancy, y	24 (6)	24 (5)	22 (4)	23 (4)
Body mass index, kg/m²	25 (6)	24 (5)	25 (6)	24 (5)
	Postmenopausal			
	Cases		Controls	
	Rapid Acetylators (n=80)	Slow Acetylators (n=105)	Rapid Acetylators (n=100)	Slow Acetylators (n=113)
Age, y	62 (7)	63 (7)	64 (8)	63 (7)
Education, y	13 (3)	12 (3)	12 (3)	12 (3)
Age at menarche, y	13 (2)	13 (2)	13 (1)	13 (2)
Age at menopause, y	47 (6)	48 (6)	47 (6)	47 (6)
Age at first pregnancy, y	25 (5)	24 (5)	23 (4)	23 (4)
Body mass index, kg/m²	26 (4)	26 (6)	26 (4)	26 (6)

*Values are expressed as mean (SD). $P<.05$ for the Student *t*-test for the difference between the means of rapid and slow acetylator cases and controls.

smoking-related variables and breast cancer risk. In premenopausal women, no clear patterns were identified by acetylator status (Table 5). Risk was elevated in rapid acetylators who had smoked 20 years before, although ORs and trend test were not statistically significant.

In postmenopausal slow acetylators, strong associations between smoking and breast cancer risk were seen (Table 5). For them, smoking 2 and 20 years previously increased breast cancer risk in a dose-dependent manner (OR [95% CI] for the highest quartile for smokers vs never-smokers, 4.4 [1.3-14.8] and 3.9 [1.4-10.8], respectively). Although breast cancer risk was elevated with total years of smoking, smoking intensity appeared more important than duration (Figure 2). Packs per average year significantly elevated breast cancer risk among postmenopausal slow acetylators, with a 3-fold risk for smoking more than 1 pack per day ($P<.01$). Total pack-years of smoking were also associated with elevated risk in this group (Table 5).

Among postmenopausal rapid acetylators, adjusted risk was not increased by smoking. For cigarettes smoked 20 years previously, there was reduced risk at the highest quartile of use (OR, 0.30; 95% CI, 0.01-0.80). Neither smoking intensity nor smoking duration was associated with increased risk in postmenopausal rapid acetylators (Figure 2).

As explained previously, a case-series analysis was used to assess associations among smoking, NAT2 status, and postmenopausal breast cancer risk. In this analysis, NAT2 status was the dependent variable in a logistic regression model in which rapid acetylator cases served as comparison group to slow acetylator cases. The resultant ORs do not reflect actual risk of breast cancer, but rather the ratio of risk for slow vs rapid acetylators. Smoking-related variables were regressed on acetylator status; the effect of smoking on risk appeared to vary according to NAT2 genotype. Heavy smokers with breast cancer were more likely to be slow acetylators (Table 6). Risk was almost 7 times greater for women who had smoked more than a pack a day 20 years previously if they were slow vs rapid acetylators (OR, 6.6; 95% CI, 1.7-25.4), and risk associated with beginning smoking at or before the age of 16 years was more than 4 times greater for slow vs rapid acetylators (OR, 4.5, 95% CI, 1.3-14.9). When pack-years smoked were evaluated, risk was almost 3 times greater for slow acetylators in the highest quartile of use.

As previously explained, we evaluated the effect of acetylator status on the association between smoking and breast cancer risk in a model designed to eliminate overall association between smoking and risk, in which postmenopausal cases and controls were matched on smoking, with an OR of 1.0 for breast cancer associated with each quartile of cigarette use. Cases and controls were then stratified by NAT2 genotype, and divergent patterns related to smoking were noted between genotypes. Smoking at each time period had a null or negative effect on breast cancer risk in rapid acetylators, while for slow acetylators, cigarette smoking was associated with increased breast cancer risk (Table 7). Risk associated with age at which smoking began was also modified by genotype; slow acetylators who began smoking at or before the age of 16 years were at highest breast cancer risk. For rapid acetylators, there was an inverse association between risk and earlier initiation of smoking. While these estimates of risk are derived from contrived subsets of data, they illustrate heterogeneity in risk by genotype of the study population in response to cigarette smoking. Mantel-Haenszel tests were performed on the unadjusted categorical data, and trend tests shown are consistent with results from the other methods of analysis. When analyses were repeated using different random-number generators to match on smoking, associations among NAT2 status, smoking, and breast cancer risk were similar.

COMMENT

Cigarette smoking appears to be a risk factor for breast cancer among postmenopausal, but not premenopausal, white women with the NAT2 slow acetylation genotype. Among slow acetylators, smoking intensity, rather than smoking duration, most greatly affected breast cancer risk. Smoking at a young age also appeared to confer risk. Among

rapid acetylators, neither intensity nor duration of smoking increased risk. When examined alone, neither NAT2 genotype nor cigarette smoking was independently associated with breast cancer risk. Differences in results for premenopausal and postmenopausal women could be related to different etiologic pathways or intrinsic differences in the disease. Also, premenopausal women, being younger, may have had fewer years of exposure to tobacco, or not enough elapsed time for the entire carcinogenic process to develop.

Laboratory studies indicate that aromatic amines are mammary mutagens and carcinogens in rodents and in humans.[29-33,53-56] Aromatic amines are bioactivated and/or detoxified by xenobiotic metabolizing enzymes, including cytochrome P4501A2 (CYP1A2) and *N*-acetyltransferases (NAT1 and NAT2). While NAT2 is the focus herein, these other enzymes might also play a role in breast cancer. Both CYP1A2 and NAT1 are polymorphic, although the genetic basis for the former is unknown. *N*-Acetylation of aromatic amines by NAT2 appears to be a detoxification step catalyzed by hepatic NAT enzymes, a pathway competing with that for *N*-oxidation by CYP1A2, whereby reactive *N*-hydroxy metabolites may enter the circulation, undergoing further activation and binding to DNA in target tissues.[34] It has been suggested that NAT1 is more active in breast tissue than NAT2,[57,58] but the importance of hepatic detoxification should not be underestimated.

The role of aromatic amines in breast cancer may be analogous to that in urinary bladder cancer.[59] Cigarette smoking is a risk factor for bladder cancer,[60] and slow acetylators have higher circulating levels of 4-aminobiphenyl-hemoglobin adducts, reflecting decreased clearance of reactive arylamine metabolites.[37,61,62] NAT2 slow acetylators have an increased bladder cancer risk, presumably because of decreased liver detoxification of aromatic amines.[39,40,63] Risk of bladder cancer associated with smoking may vary by NAT2 status,[64] although results have been inconsistent.[65,66] Animal studies indicate that mammary and bladder tissues have similar sensitivities to reactive intermediates.[53] Aromatic amines are rodent mammary carcinogens if activated in the liver.[32] Thus, slow acetylators may have less capacity to detoxify aromatic amines, leading to an increased concentration of reactive intermediates. In the breast, further activation may occur,[57,67] resulting in promutagenic carcinogen-DNA adducts and carcinogenesis.

Table 4.—*N*-Acetyltransferase 2 Polymorphisms and Smoking Characteristics for Cases and Controls: Western New York Breast Cancer Study, 1986 to 1991*

	Premenopausal			
	Cases		Controls	
Characteristic	Rapid Acetylators	Slow Acetylators	Rapid Acetylators	Slow Acetylators
Daily cigarettes 2 y ago	22 (7)	22 (15)	23 (16)	25 (15)
Daily cigarettes 20 y ago	18 (7)	17 (11)	18 (12)	20 (14)
Age smoking began, y	17 (5)	18 (4)	16 (3)	17 (4)
Total duration of smoking, y	22 (9)	19 (9)	23 (8)	22 (10)
Packs per average year	272 (147)	288 (216)	292 (179)	336 (244)
Pack-years	19 (14)	19 (17)	21 (16)	25 (23)
	Postmenopausal			
	Cases		Controls	
	Rapid Acetylators	Slow Acetylators	Rapid Acetylators	Slow Acetylators
Daily cigarettes 2 y ago	21 (11)	22 (10)	19 (9)	19 (13)
Daily cigarettes 20 y ago	16 (8)†	24 (12)†	21 (12)	19 (14)
Age smoking began, y	21 (6)	20 (5)	21 (9)	21 (9)
Total duration of smoking, y	30 (13)	33 (13)	33 (13)	31 (14)
Packs per average year	285 (156)†	397 (204)†	276 (170)	290 (207)
Pack-years	29 (18)†	43 (27)†	27 (20)	29 (22)

*Values are expressed as mean (SD).
†$P<.01$ for the Student t test for the difference between the means of rapid and slow acetylator cases and controls.

Table 5.—Breast Cancer Risk and Cigarette Smoking by Acetylator Genotype: Western New York Breast Cancer Study, 1986 to 1991*

	Premenopausal						Postmenopausal					
	Rapid Acetylators			Slow Acetylators			Rapid Acetylators			Slow Acetylators		
Quartile	Case	Control	OR (95% CI)	Case	Control	OR (95% CI)	Case	Control	OR (95% CI)	Case	Control	OR (95% CI)
Daily cigarettes 2 y ago												
None	18	25	1.0 ...	27	31	1.0 ...	43	50	1.0 ...	41	59	1.0 ...
≤15	2	5	0.7 (0.1-4.6)	6	7	0.6 (0.1-2.5)	6	7	1.8 (0.5-6.5)	6	12	0.8 (0.3-2.5)
16-20	10	4	4.5 (1.0-20.6)	12	4	3.5 (0.9-14.2)	8	11	1.0 (0.3-3.0)	21	11	3.2 (1.3-7.8)
>20	4	5	0.7 (0.1-4.2)	4	10	0.4 (0.1-1.7)	5	3	2.1 (0.4-10.4)	11	5	4.4 (1.3-14.8)
P for trend		.72			.49			.51			<.01	
Cigarettes 20 y ago												
None	18	25	1.0 ...	27	31	1.0 ...	43	50	1.0 ...	41	59	1.0 ...
≤15	8	8	1.6 (0.5-5.5)	12	10	0.9 (0.3-2.7)	13	12	1.5 (0.6-3.8)	10	19	0.9 (0.4-2.2)
16-20	10	10	2.2 (0.7-6.8)	14	12	1.1 (0.4-3.2)	13	20	1.1 (0.5-2.6)	23	18	2.3 (1.0-5.0)
>20	2	2	2.6 (0.3-22.3)	4	3	1.2 (0.2-6.3)	3	11	0.3 (0.1-0.8)	17	7	3.9 (1.4-10.8)
P for trend		.35			.63			.21			<.01	
Pack-years												
None	18	25	1.0 ...	27	31	1.0 ...	43	50	1.0 ...	41	59	1.0 ...
≤183	9	7	1.8 (0.5-6.5)	13	7	1.7 (0.5-5.1)	4	12	0.4 (0.1-1.6)	5	9	0.9 (0.3-3.0)
184-365	11	7	2.5 (0.7-8.6)	10	10	0.8 (0.3-2.4)	10	10	1.5 (0.5-4.2)	10	14	1.1 (0.4-2.7)
>365	10	7	2.1 (0.5-7.9)	11	10	1.2 (0.4-3.8)	14	23	0.9 (0.4-2.1)	36	23	2.8 (1.4-5.5)
P for trend		.51			.75			.98			<.01	

*Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer, and age at menopause among postmenopausal women. Included in each analysis are lifetime nonsmokers (referent [ellipses]) and those who were smoking during that time period; excluded are those who quit smoking before that time period. *P* for trend was calculated from logistic regression with the independent variable as a continuous variable.

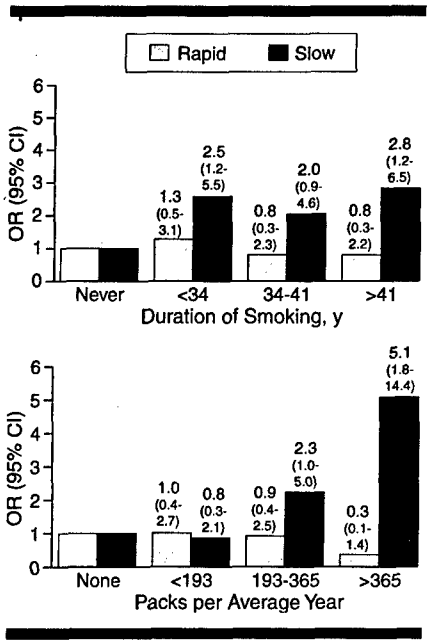

Figure 2.—Odds ratios (ORs) and 95% confidence intervals (CIs) for breast cancer by duration (top) and intensity (bottom) of cigarette smoking among postmenopausal women with rapid and slow acetylation genotypes: Western New York Breast Cancer Study, 1986-1991.

Table 6.—Case Series Analysis of *N*-Acetyltransferase 2 Status and Cigarette Smoking Among Postmenopausal Women: Western New York Breast Cancer Study, 1986 to 1991*

Quartile	No. of Slow Acetylators	No. of Rapid Acetylators	OR (95% CI)	*P* for Trend
Daily cigarettes 2 y ago				
None	41	43	1.0 ...	.05
≤15	6	6	0.8 (0.2-2.8)	
16-20	21	8	2.7 (1.0-6.9)	
>20	11	5	2.5 (0.7-8.3)	
Daily cigarettes 20 y ago				
None	41	43	1.0 ...	<.01
≤15	10	13	0.7 (0.3-1.9)	
16-20	23	13	1.6 (0.7-3.8)	
>20	17	3	6.6 (1.7-25.4)	
Duration of smoking, y				
Never smoked	41	43	1.0 ...	.10
≤18	10	8	1.5 (0.5-4.2)	
19-25	8	3	2.6 (0.6-10.8)	
>25	46	25	1.9 (1.0-3.6)	
Pack-years				
None	41	43	1.0 ...	<.01
≤15	5	4	1.1 (0.3-4.8)	
16-20	10	10	1.0 (0.3-2.7)	
>20	36	4	2.7 (1.2-5.8)	
Age at smoking initiation, y				
Never smoked	41	43	1.0 ...	.01
>18	31	25	1.2 (0.6-2.5)	
17-18	17	8	2.2 (0.7-7.3)	
≤16	16	4	4.5 (1.3-14.9)	

*Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, and family history of breast cancer, with rapid acetylators as the comparison group. Resultant ORs do not reflect actual risk of breast cancer, but rather the ratio of risk for slow vs rapid acetylators. Included in each analysis are lifetime nonsmokers (referent [ellipses]) and those who were smoking during that time period; excluded are those who quit smoking before that time period. *P* for trend was calculated from logistic regression, with the independent variable as a continuous variable.

Table 7.—Smoking-Matched Case-Control Analysis of Postmenopausal Breast Cancer Risk and Cigarette Smoking by Acetylator Genotype: Western New York Breast Cancer Study, 1986 to 1991*

	Rapid Acetylators			Slow Acetylators		
Quartile	Cases	Controls	OR (95% CI)	Cases	Controls	(95% CI)
Daily cigarettes 2 y ago						
None	43	42	1.0 ...	41	42	1.0 ...
≤15	6	6	1.9 (0.5-7.1)	6	6	1.1 (0.3-3.8)
16-20	6	11	0.6 (0.2-2.0)	16	11	1.7 (0.6-4.3)
>20	2	3	0.5 (0.1-3.6)	6	5	1.6 (0.4-5.9)
P for trend		.28			.44	
Daily cigarettes 20 y ago						
None	43	36	1.0 ...	41	48	1.0 ...
≤15	13	10	1.3 (0.4-3.7)	10	13	1.0 (0.4-2.7)
16-20	13	20	0.8 (0.3-1.9)	23	16	2.1 (0.9-4.7)
>20	3	11	0.2 (0.04-0.8)	15	7	2.6 (0.9-7.4)
P for trend		.02			<.01	
Age at smoking initiation, y						
Never smoked	43	40	1.0 ...	41	44	1.0 ...
>18	19	20	1.1 (0.5-2.6)	27	26	1.0 (0.6-2.7)
17-18	8	11	0.6 (0.2-1.7)	16	13	1.4 (0.6-3.5)
≤16	4	13	0.2 (0.01-1.0)	16	7	3.2 (1.1-9.1)
P for trend		<.05			.09	

*Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, and family history of breast cancer. Included in each analysis are lifetime nonsmokers (referent [ellipses]) and those who were smoking during that time period; excluded are those who quit smoking before that time period. *P* for trend was calculated from Mantel-Haenszel tests with unadjusted categorical data.

The case-series and case-control analyses reported herein indicate that effects may be strongest for smoking at an earlier age (ie, age at which smoking began, number of cigarettes smoked 20 years previously), consistent with the hypothesis that environmental insults may be most deleterious during breast development.[68] The apparently stronger effects of heavier smoking, rather than duration of smoking, may also reflect carcinogenic doses administered at an earlier age. Risk associated with smoking 2 years previously may be increased only because heavy smoking occurred at a younger age, and risk relates to exposure earlier, rather than later, in life. Cigarette smoking is clearly a risk factor for lung and bladder cancer, and genetic polymorphisms in carcinogen-metabolizing genes may increase risk at low exposures.[37,69] For breast cancer, the association is less straightforward.

Among rapid acetylators, there was weak indication that smoking at an early age and smoking more than 1 pack per day 20 years previously reduced breast cancer risk in comparison to that of slow acetylators. In women who are rapid acetylators, carcinogenic aromatic amines may be quickly detoxified and excreted, and other smoke components may have inverse risk effects. It has been suggested that smoking may have antiestrogenic effects, thus decreasing breast cancer risk.[70-72] In rapid acetylators, induction of other enzyme activity by smoking may lead to increased metabolism, and therefore to reduction of circulating estradiol. In rapid acetylators, this antiestrogenic effect may override carcinogenic potential, reducing breast cancer risk. Dual effects of these opposing forces may account for previous failures to observe associations between smoking and breast cancer risk. This is speculation, and the

inconsistent findings for rapid acetylators may be due to chance.

Other tobacco smoke constituents, such as *N*-nitrosamines and polycyclic aromatic hydrocarbons, may be related to breast cancer. We studied polymorphisms related to polycyclic aromatic hydrocarbon biotransformation, *CYP1A1* and *GSTM1*, regarding cigarette smoking and postmenopausal breast cancer risk.[73] Neither *CYP1A1* nor *GSTM1* was independently associated with risk, but light smokers with the *CYP1A1* minor allele had increased risk.

Potential sources of bias in these data exist, foremost of which is selection bias. Most case nonparticipation was due to physicians' refusals to allow contact with their patients (72%), and postmenopausal nonparticipants were, on average, about 3 years older than participants. The most ill patients may not have been included, limiting generalizability. Among controls, a sample refusing interview (n=117) was compared with a sample of participants (n=372) in a telephone interview prior to data collection. No differences in meat, vegetable, and fruit consumption or in number of cigarettes smoked were found. Thus, nonresponse among controls may not have been related to exposure. Other potential sources of bias in control selection include random selection of controls younger than 65 years from lists of driver's license holders. Among cases, some women may not drive and may be different from selected controls, but more than 90% of women in this state are licensed to drive. Because controls were drawn from the community and not screened for undetected breast cancer, a small number of controls may have had breast cancer.

All of these sources of bias may affect risk estimates. In estimating risk associated with smoking in slow acetylators, there may have been some overestimation of true risk due to selection bias, since the proportion of nonsmokers was greater in controls consenting to phlebotomy than in the study group. We thus corroborated findings using case-series analysis and generating smoking-matched data subsets in which the ORs were lower than in initial case-control analyses and more likely estimate true risk. We concluded that, while point estimates of risk may be biased, such bias does not explain the marked difference in smoking risk patterns among slow vs rapid acetylators. While biases may affect risk point estimates, it is unlikely that they could affect heterogeneity of response by NAT2 acetylation status, creating substantial differences in associations between smoking and risk. Nor is it likely that biases were associated with genotype.

We stratified women by susceptible subgroups and examined the role of genotype in susceptibility to an exogenous exposure, cigarette smoking, not heretofore consistently shown to be related to breast cancer risk. In our data, 57% of the postmenopausal women studied were slow acetylators, similar to findings from previous studies.[37,74] Among these women, it appeared that smoking, particularly at an early age, increased breast cancer risk. Slow NAT2 phenotype prevalence varies worldwide, estimated present in 10% to 20% of Asians, 35% of African Americans, 65% to 90% of individuals of Middle Eastern descent, and about 55% of whites.[61,74-77] Ethnic distribution of NAT2 genotype, in relation to exposure to aromatic amines, could explain, in part, the wide geographic variability in breast cancer incidence.

In these data, there was some weak indication of a negative association between breast cancer risk among women with the rapid NAT2 genotype, which does not suggest that smoking is beneficial. Smoking cessation should be a goal for all women.

These findings require replication. If further investigations reveal similar associations, a portion of unexplained breast cancer etiology will have been elucidated. Our data suggest that, for more than half the white female population of the United States, avoidance of breast cancer may be yet another motivation for avoiding cigarette smoking, particularly at an early age.

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APPENDIX C -Breast cancer risk, meat consumption and N-acetyltransferase (NAT2) genetic polymorphisms

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BREAST CANCER RISK, MEAT CONSUMPTION AND *N*-ACETYLTRANSFERASE (*NAT2*) GENETIC POLYMORPHISMS

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Although inconsistencies exist, some studies have shown that meat consumption is associated with breast cancer risk. Several heterocyclic amines (HAs), formed in the cooking of meats, are mammary carcinogens in laboratory models. HAs are activated by polymorphic *N*-acetyltransferase (NAT2) and rapid NAT2 activity may increase risk associated with HAs. We investigated whether ingestion of meat, chicken and fish, as well as particular concentrated sources of HAs, was associated with breast cancer risk, and if *NAT2* genotype modified risk. Caucasian women with incident breast cancer (n = 740) and community controls (n = 810) were interviewed and administered a food frequency questionnaire. A subset of these women (n = 793) provided a blood sample. Polymerase chain reaction and restriction fragment length polymorphism analyses were used to determine *NAT2* genotype. Consumption of red meats, as well as an index of concentrated sources of HAs, was not associated with increased breast cancer risk, nor did risk vary by *NAT2* genotype. In post-menopausal women, higher fish consumption was inversely associated with risk (odds ratio = 0.7; 95% confidence interval, 0.4–1.0); among pre-menopausal women, there was the suggestion of inverse associations between risk and pork and chicken intake. Our results suggest that consumption of meats and other concentrated sources of HAs is not associated with increased breast cancer risk. However, due to the strong biologic plausibility for a role of some HAs in mammary carcinogenesis, and the likely measurement error in evaluation of sources of HAs in this study, further studies of these possible relationships are warranted. *Int. J. Cancer* 75:825–830, 1998.
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The incidence of breast cancer varies widely by geographic region (Parkin and Muir, 1992), and it has been suggested that variability in diet, particularly intake of dietary fat and protein, may be related to this disparity in breast cancer rates (Prentice *et al.*, 1988), although the majority of epidemiologic studies have not supported this association (Hunter and Willett, 1996). Studies of the consumption of animal products, particularly meat, have also yielded inconsistent results, although a meta-analysis of 5 cohort and 12 case-control studies by Boyd *et al.*, (1993) did reveal a summary relative risk of of 1.54 (95% CI 1.31–1.82) associated with consumption of red meat (Boyd *et al.*, 1993).

The assessment of meat as a risk factor for breast cancer has focused primarily on its role as a source of dietary fat or animal protein. However, in 3 studies it was found that consumption of meat, when controlling for total fat or protein, significantly increased breast cancer risk (Toniolo *et al.*, 1994, Ronco *et al.*, 1996, De Stefani *et al.*, 1997). It is possible that if meat consumption does play a role in breast cancer etiology, it may be due to its being a source of mutagens and/or carcinogens, such as heterocyclic amines, which are potent mammary mutagens and carcinogens in rodent models (Snyderwine, 1994). Disparate cooking methods in different populations or survey instruments inadequate to assess concentrated sources of heterocyclic amines (HAs) may be related to these inconsistencies in results across studies.

Metabolism of heterocyclic and aromatic amines varies among individuals, depending, in part, on polymorphisms in genes involved in xenobiotic metabolism, such as *N*-acetyltransferases NAT1 and NAT2 and cytochrome P4501A2 (CYP1A2) (Lang *et al.*, 1994). Several polymorphic sites have been identified at the *NAT2* locus, and result in decreased *N*-acetyltransferase activity (Bell *et al.*, 1993). Slow NAT2 acetylation of aromatic amines is associated with increased risk for bladder cancer (Cartwright, 1984) and may increase post-menopausal breast cancer risk associated with cigarette smoking (Ambrosone *et al.*, 1996). HAs appear to be poor substrates for *N*-acetylation in the liver, however, and rapid *O*-acetylation of the activated metabolites by NAT2 in the target tissue appears to be associated with increased risk of colon cancer related to consumption of red meat (Welfare *et al.*, 1997; Lang *et al.*, 1994). If HAs are etiologic agents in human breast carcinogenesis, it is plausible that rapid activation by NAT2 would also be associated with increased breast cancer risk.

The purpose of our analyses was 3-fold: 1) we sought to evaluate relationships between breast cancer risk and consumption of meats, poultry and fish in pre- and post-menopausal women; 2) we were interested in determining whether risk associated with meat consumption could be related to dietary HAs, as measured by consumption of products known to be concentrated sources of these carcinogens; and 3) we wanted to determine whether polymorphisms in *NAT2* might modify the association between breast cancer risk and consumption of sources of heterocyclic amines.

MATERIAL AND METHODS

Study population

This study population and research methodology have been described in detail previously (Freudenheim *et al.*, 1996; Ambrosone *et al.*, 1996; Graham *et al.*, 1991). Briefly, cases were women diagnosed with incident, primary, histologically confirmed breast cancer, identified from all the major hospitals in Erie and Niagara counties; included were women ranging in age from 40 to 85.

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Women under age 50 were considered post-menopausal if they had ceased menstruation because of natural menopause, bilateral oopherectomy, or irradiation to the ovaries; all others were considered pre-menopausal. Women 50 years of age and over were considered post-menopausal if they were no longer menstruating. Cases were interviewed, on average, within 2 months of diagnosis. Controls under 65 years of age were randomly selected from the New York State Motor Vehicle Registry, and those 65 and over were identified from Health Care Finance Administration lists. Of pre-menopausal women contacted, 66% of eligible cases (n = 301) and 62% of eligible controls (n = 316) participated, and of post-menopausal women, 54% of cases (n = 439) and 44% of controls (n = 494) participated. Controls were frequency-matched to cases by age and county of residence. The protocol for the study was reviewed by the Institutional Review Board of the State University of New York at Buffalo and each participating hospital, and informed consent was received from all participants. Cases and controls were interviewed in person by trained interviewers, with an in-depth food frequency questionnaire regarding usual diet 2 years prior to the interview, including frequency of consumption and usual portion size of over 300 specific foods. Reproductive, medical and family histories were obtained, as well as lifetime tobacco and alcohol histories. Of the women interviewed, approximately 45% of pre-menopausal and 63% of post-menopausal women agreed to have blood drawn for research purposes.

Analytic methodology

An extensive food frequency questionnaire was administered, assessing usual intake 2 years prior to the interview. Using food models, women were questioned about usual dietary intake 2 years prior to the interview, including quantity and frequency of intake, seasonal intake and food preparation. Grams of meats per day were computed by multiplying frequency of consumption by portion size, estimated by food models. Participants were asked about portion size and frequency of consumption of steak, round steak, hamburger patties, ground beef, other beef, including roasts and stews, veal, lamb and beef liver. From this information, usual grams of consumption of each item were calculated and items were grouped to create a beef index. A pork index was based on queries regarding intake of pork roast, chops and spareribs. A processed meats index, including ham, hot dogs, sausages, bacon and cold cuts was also assessed. A poultry index included chicken and turkey. The fish index included fresh or frozen fish, canned fish, shrimp and other shellfish. In addition to frequency of consumption and usual portion size of various types of meat, women were also asked how frequently they used gravy made from pan drippings or fried foods in bacon grease. We also evaluated associations between risk and grams consumed per month of bacon, breakfast sausages and gravy made from pan drippings, all concentrated sources of HAs, particularly PhIP (Murray *et al.*, 1993). Data were not available on how well done the meat consumed was cooked, which is another indicator of exposure to HAs.

Risks for pre- and post-menopausal women were examined separately, based on variability in some risk factors and the possibility that breast cancer may be different diseases in the 2 groups. Furthermore, mean levels of intake of certain meats varied significantly between the 2 groups. Quartiles of intake of types of meats were based on approximately uniform distribution in controls. Odds ratios (OR) with 95% confidence intervals (CI) were calculated by unconditional logistic regression for each category of the risk variables, with the lowest intake quartile as the referent category. The p values for trend were the levels of significance of the beta coefficient for each independent variable as a continuous variable in the logistic regression model with the relevant adjusting variables. ORs were adjusted for putative breast cancer risk factors including age, education, body mass index (BMI), age at menarche, age at first pregnancy, family history of breast cancer and age at menopause for post-menopausal women. BMI was computed as weight(kg)/height(m)^2, where weight was as reported for 2 years prior to the interview, and family history was defined as the presence of breast cancer in a mother and/or sister. Because there may be a tendency for fish and poultry eaters also to consume more fruits and vegetables, and because some components of fruits and vegetables, which were associated with reduced risk in these data (Freudenheim *et al.*, 1996), may reduce mutagenic activity, an additional model was employed, adjusting for total fruit and vegetable consumption. To evaluate variable risk in relation to consumption of sources of HAs, cases and controls were stratified by acetylator status and the relationship between breast cancer risk and these foods was assessed within rapid and slow acetylator groups. Sample size for these latter determinations was restricted to those who provided a blood sample and for whom NAT2 data were available. This included 118 and 114 pre-menopausal cases and controls, and 185 and 213 post-menopausal cases and controls.

NAT2 *genotyping*

Blood specimens were collected, serum was separated and blood clots were stored at −70°C. Methods for DNA extraction from clots and determination of *NAT2* genotype have been described previously (Ambrosone *et al.*, 1996). Briefly, DNA was extracted and amplified by polymerase chain reaction (PCR) in the presence of primers specific for *NAT2* (Bell *et al.*, 1993). An aliquot (18 µl) was then subjected to restriction fragment length polymorphism (RFLP) analysis for the $C^{481}T$ (KpnI; New England Biolabs, Beverly, MA), $G^{590}A$ (TaqI, New England Biolabs) and the $G^{857}A$ (BamHI, New England Biolabs) polymorphisms. Individuals were classified as genotypically determined rapid acetylator (carrying 0 or 1 slow acetylator mutation) or slow acetylator (individuals with 2 slow acetylator mutations) (Lin *et al.*, 1993; De Stefani *et al.*, 1994). Assays were performed in duplicate and were interpreted by 2 reviewers who were blinded to case-control status.

RESULTS

Table I shows reported mean values of consumption of various meats for all pre- and post-menopausal women. Pre-menopausal controls consumed significantly more pork and fish than cases. In the interpretation of these reports, it is important to note that the diet assessment instrument used is a well-established tool for qualitative assessment of intake and that quantitative assessment may be less accurate. There were no significant differences in means for any of the variables tested among post-menopausal women. Associations between breast cancer risk and quartiles of consumption of various meats for pre- and post-menopausal women are shown in Tables II and III. Total calories consumed were not related to breast cancer risk in these data, and the addition of this variable to the model did not significantly alter estimates of risk. Models adjusted for cigarette smoking, found to increase risk among post-menopausal women with slow *NAT2* genotype in these data, also did not differ significantly from unadjusted. For pre-menopausal women, there was no increased risk associated with consumption of beef, processed meats, pork, chicken or fish (Table II). In fact, there were inverse associations between breast cancer risk and consumption of pork, chicken and fish, although of borderline significance. However, the association between fish and chicken consumption and breast cancer risk was weaker after adjustment for fruit and vegetables.

Among post-menopausal women, there was no increase in breast cancer risk associated with higher consumption of beef, pork or processed meats (Table III). Both chicken and fish consumption were inversely associated with risk of post-menopausal breast cancer (4th quartile ORs and 95% CIs, respectively, 0.7, 0.5–1.0, and 0.6, 0.4–0.9). These relationships remained when adjustment was made for total fruit and vegetable consumption.

Tables IV and V present analyses for the subset of women who provided blood specimens. When associations were assessed within categories of rapid and slow acetylators, there were no clear

TABLE I – CHARACTERISTICS OF CASES AND CONTROLS: WESTERN NEW YORK DIET STUDY, 1986–1991[1]

	Pre-menopausal		Post-menopausal	
	Cases (n = 301)	Controls (n = 316)	Cases (n = 439)	Controls (n = 494)
Age (years)	46 (4)	46 (4)	64 (8)	63 (8)
Education (years)	14 (3)	14 (3)	12 (3)	12 (3)
Beef intake (g/day)	60 (47)	60 (39)	52 (44)	51 (37)
Pork intake (g/day)	12 (9)[2]	14 (11)[2]	11 (10)	11 (10)
Processed meat intake[3] (g/day)	39 (41)	37 (31)	31 (30)	31 (31)
Poultry intake (g/day)	31 (22)	34 (24)	23 (17)	25 (21)
Fish intake (g/day)	27 (15)[2]	32 (28)[2]	25 (20)	28 (22)
Ratio of red meat/poultry and fish	1.5 (1.3)	1.5 (1.3)	2.8 (4.5)	2.4 (2.3)

[1]Values are expressed as mean (standard deviation).–[2]$p < 0.01$, Student's t-test for difference between means of cases and controls.–[3]Includes bacon, breakfast sausages, ham, hot dogs, bologna and other cold cuts.

TABLE II – MEAT CONSUMPTION (g/day) AND PREMENOPAUSAL BREAST CANCER RISK: WESTERN NEW YORK DIET STUDY, 1986–1991

Quartiles	Case (number)	Control (number)	OR (CI)[1]	OR (CI)[2]
Beef (g/day)				
<33	74	82	1.0	1.0
33–51	85	77	1.3 (0.8–2.0)	1.3 (0.8–2.1)
51–78	68	78	1.0 (0.6–1.5)	1.0 (0.6–1.6)
>78	74	79	1.1 (0.7–1.7)	1.2 (0.8–1.9)
			p (trend) = 0.76	p (trend) = 0.3
Pork (g/day)				
<6	92	82	1.0	1.0
6–10	70	79	0.8 (0.5–1.2)	0.8 (0.5–1.2)
10–20	91	82	1.0 (0.6–1.5)	1.0 (0.6–1.5)
>20	48	73	0.6 (0.4–1.0)	0.6 (0.4–1.0)
			p (trend) = 0.02	p (trend) = 0.05
Processed meats (g/day)[3]				
<14	65	80	1.0	1.0
14–29	94	79	1.6 (1.0–2.5)	1.5 (1.0–2.4)
29–48	60	78	1.0 (0.6–1.6)	1.0 (0.6–1.6)
>48	82	79	1.4 (0.8–2.2)	1.4 (0.9–2.3)
			p (trend) = 0.3	p (trend) = 0.09
Poultry (g/day)				
<19	95	79	1.0	1.0
19–28	66	79	0.7 (0.4–1.1)	0.7 (0.4–1.1)
28–43	84	79	0.8 (0.5–1.3)	0.9 (0.6–1.4)
>43	56	79	0.6 (0.4–0.9)	0.7 (0.4–1.1)
			p (trend) = 0.2	p (trend) = 0.6
Fish (g/day)				
<15	83	80	1.0	1.0
15–26	85	79	1.1 (0.7–1.7)	1.1 (0.7–1.8)
26–38	71	75	0.9 (0.6–1.5)	1.0 (0.6–1.6)
>38	62	82	0.8 (0.5–1.2)	0.9 (0.6–1.5)
			p (trend) = 0.03	p (trend) = 0.2
Ratio of red meat to chicken and fish				
<0.7	71	80	1.0	1.0
0.7–1.2	70	85	0.9 (0.6–1.4)	0.9 (0.5–1.4)
1.2–1.8	72	76	1.1 (0.7–1.7)	1.0 (0.6–1.7)
>1.8	88	75	1.4 (0.9–2.2)	1.3 (0.8–2.0)
			p (trend) = 0.5	p (trend) = 0.8

[1]Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer.–[2]Adjusted for the variables listed above, and total fruits and vegetables.–[3]Includes bacon, breakfast sausages, ham, hot dogs, bologna and other cold cuts.

associations between risk and consumption of beef, pork, chicken, fish or processed meats among pre- or postmenopausal women by genotype (data not shown). Evaluation of risk associated with consumption of foods that are concentrated sources of heterocyclic amines (bacon, gravy, breakfast sausages) also revealed no clear or significant associations, when groups were evaluated all together, or when stratified by *NAT2* genotype. Associations with risk were also evaluated by frequency of consumption of various meats that were fried or grilled, but no effect was observed (data not shown).

DISCUSSION

In this case-control study of diet and breast cancer, we found that, in general, consumption of meats was not associated with increased breast cancer risk for pre- or post-menopausal women. Increased intake of fresh, frozen or canned fish, as well as poultry, appeared to be associated with decreased risk among post-menopausal women. Among pre-menopausal women, there was a suggestion of a slight inverse association with pork consumption.

TABLE III – MEAT CONSUMPTION (g/day) AND POST-MENOPAUSAL BREAST CANCER RISK: WESTERN NEW YORK DIET STUDY, 1986–1991

Quartiles	Case (number)	Control (number)	OR (CI)[1]	OR (CI)[2]
Beef (g/day)				
<28	113	123	1.0	1.0
28–45	132	121	1.1 (0.8–1.7)	1.2 (0.8–1.7)
45–62	78	122	0.7 (0.5–1.0)	0.7 (0.5–1.0)
>62	116	128	0.9 (0.6–1.3)	1.0 (0.7–1.4)
			p (trend) = 0.5	*p* (trend) = 0.3
Pork (g/day)				
<4	96	98	1.0	1.0
4–8	118	137	0.9 (0.6–1.3)	0.9 (0.6–1.3)
8–15	128	133	1.0 (0.7–1.4)	1.0 (0.7–1.4)
>15	97	126	0.7 (0.5–1.1)	0.8 (0.5–1.2)
			p (trend) = 0.3	*p* (trend) = 0.5
Processed meats (g/day)[3]				
<11	101	122	1.0	1.0
11–22	117	126	1.1 (0.8–1.6)	1.1 (0.8–1.6)
22–40	112	124	1.1 (0.7–1.6)	1.1 (0.8–1.6)
>40	109	122	1.0 (0.7–1.5)	1.1 (0.7–1.6)
			p (trend) = 0.9	*p* (trend) = 0.5
Poultry (g/day)				
<12	126	120	1.0	1.0
12–19	119	125	0.8 (0.6–1.2)	0.8 (0.6–1.2)
19–30	80	122	0.5 (0.4–0.8)	0.5 (0.4–0.8)
>30	114	127	0.7 (0.5–1.0)	0.8 (0.5–1.1)
			p (trend) = 0.01	*p* (trend) = 0.04
Fish (g/day)				
<13	129	124	1.0	1.0
13–23	117	131	0.9 (0.6–1.3)	0.9 (0.7–1.3)
23–38	112	120	0.8 (0.6–1.2)	0.9 (0.6–1.3)
>38	81	119	0.6 (0.4–0.9)	0.7 (0.4–1.0)
			p (trend) = 0.03	*p* (trend) = 0.2
Ratio of red meat to chicken and fish				
<1.2	107	130	1.0	1.0
1.2–1.9	94	125	0.9 (0.6–1.4)	0.9 (0.6–1.3)
1.9–2.8	94	109	1.0 (0.7–1.5)	1.0 (0.7–1.4)
>2.8	144	130	1.4 (1.0–2.1)	1.3 (0.9–1.9)
			p (trend) = 0.1	*p* (trend) = 0.1

[1]Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, family history of breast cancer.–[2]Adjusted for the variables listed above, and total fruits and vegetables.–[3]Includes bacon, breakfast sausages, ham, hot dogs, bologna and other cold cuts.

TABLE IV – CONSUMPTION OF CONCENTRATED SOURCES OF HETEROCYCLIC AMINES (BACON, BREAKFAST SAUSAGE, GRAVY) AND BREAST CANCER RISK

Sources of heterocyclic amines (g/month)	Pre-menopausal			Post-menopausal		
	Cases	Controls	OR (CI)[1]	Cases	Controls	OR (CI)[1]
All women with genetic data						
<58	25	28	1.0	45	53	1.0
58–149	26	31	0.8 (0.4–1.9)	39	55	0.8 (0.4–1.4)
149–464	45	27	2.0 (0.9–4.3)	43	59	0.7 (0.4–1.3)
>464	22	28	0.9 (0.4–2.1)	58	46	1.4 (0.8–2.5)
NAT2 rapid						
<58	11	9	1.0	25	31	1.0
58–149	9	13	0.8 (0.2–3.1)	20	31	0.8 (0.3–1.9)
149–464	21	11	2.7 (0.7–9.9)	29	29	0.3 (0.1–0.9)
>464	10	16	0.9 (0.2–3.4)	31	22	1.0 (0.4–2.6)
NAT2 slow						
<58	14	19	1.0	20	22	1.0
58–149	17	18	0.9 (0.3–2.8)	19	24	0.9 (0.4–2.0)
149–464	24	16	1.8 (0.6–5.4)	14	30	1.2 (0.5–2.6)
>464	12	12	1.2 (0.3–3.9)	27	24	1.9 (0.9–4.3)

[1]Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer, fruit and vegetable consumption and age at menopause for post-menopausal women.

In studying associations between dietary sources of heterocyclic amines and breast cancer risk, we had extensive data regarding portion size and method of cooking for a number of meats. However, no data were available on how well-done the meat was cooked. Because a major determinant of HAs appears to be how well the meat is cooked (Sinha *et al.*I 1995), it is possible that our

TABLE V – FREQUENCY OF CONSUMPTION OF GRAVY MADE FROM PAN DRIPPINGS AND FOODS FRIED IN BACON GREASE

	Pre-menopausal			Post-menopausal		
	Cases	Controls	OR (CI)[1]	Cases	Controls	OR (CI)[1]
Frequency of gravy consumption						
All women with genetic data						
Never	10	10	1.0	23	34	1.0
<once/month	27	35	1.3 (0.4–3.6)	40	52	1.0 (0.5–2.1)
1–3 times/month	41	25	0.7 (0.4–1.6)	49	60	1.1 (0.5–2.1)
Once/week–daily	40	44	1.9 (0.9–3.9)	73	67	1.6 (0.8–3.0)
NAT2 rapid						
Never	5	2	1.0	9	12	1.0
<once/month	11	14	3.8 (0.5–27.1)	21	28	0.9 (0.3–2.7)
1–3 times/month	19	9	0.7 (0.2–2.2)	19	26	0.8 (0.3–2.6)
Once/week–daily	16	24	2.6 (0.8–8.7)	31	34	1.0 (0.3–3.0)
NAT2 slow						
Never	5	8	1.0	14	22	1.0
<once/month	16	21	0.6 (0.2–2.5)	19	24	1.0 (4–2.7)
1–3 times/month	22	16	0.7 (0.2–1.8)	30	34	1.3 (0.5–3.0)
Once/week–daily	24	20	1.2 (0.4–3.1)	42	33	2.1 (0.9–5.0)
Frequency of consumption of foods fried in bacon fat						
All women with genetic data						
Never	89	83	1.0	131	158	1.0
<once/month	16	17	0.8 (0.4–1.8)	22	28	0.9 (0.5–1.7)
1–3 times/month	6	7	0.7 (0.2–2.3)	20	16	1.6 (0.8–3.4)
Once/week–daily	7	7	1.2 (0.4–3.9)	12	9	1.8 (0.7–4.5)
NAT2 rapid						
Never	38	31	1.0	58	75	1.0
<once/month	7	11	0.6 (0.2–1.8)	9	14	0.6 (0.2–1.6)
1–3 times/month	2	3	0.7 (0.1–4.9)	10	8	1.9 (0.6–5.5)
Once/week–daily	4	4	1.1 (0.2–5.4)	3	2	1.7 (0.2–13.7)
NAT2 slow						
Never	51	52	1.0	73	83	1.0
<once/month	9	6	1.4 (0.4–4.8)	13	14	1.1 (0.5–2.6)
1–3 times/month	4	4	0.9 (0.2–4.0)	10	8	1.5 (0.5–4.3)
Once/week–daily	3	3	1.3 (0.2–7.6)	9	7	1.6 (0.5–4.7)

[1]Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer, fruit and vegetable consumption and age at menopause for post-menopausal women.

measurement of sources of HAs by grams of meats consumed was too crude to assess dietary intake of HAs accurately. However, bacon, breakfast sausages, and gravy made from pan drippings are documented sources of HAs, and these foods were also not associated with breast cancer risk.

We had hypothesized that consumption of all sources of HAs, including fish, chicken and pork, could be related to breast cancer risk. Reasons for the slight inverse associations between pork (pre-menopausal women) and chicken (post-menopausal women) are unknown, although there is the possibility that they are due to chance, or to biased reports. However, the finding of reduced risk with fish consumption among post-menopausal women is supported by some human and animal data. Few epidemiologic studies have investigated the association of breast cancer risk with fish consumption. Some case-control studies did find that fish consumption, particularly poached fish, was associated with decreased risk (Hirose *et al.*, 1995; Landa *et al.*, 1994; Vatten *et al.*, 1990; De Stefani *et al.*, 1997), and ecologic studies show that populations with high fish consumption have lower breast cancer rates (Caygill *et al.*, 1996; Kaizer *et al.*, 1989; Lund and Bonaa, 1993). Additionally, laboratory studies in rodent models and with human mammary epithelial cells have shown that dietary omega-3 fatty acids, found in fish oil, suppress growth of carcinomas (Rose and Connolly, 1993). Fish that is pan-fried or broiled may be a source of HAs, however, which may counteract some of the anticarcinogenic effects that fish oil may have. Further investigations of breast cancer risk and fish consumption, particularly by method of cooking, may elucidate these issues.

The observation of a stronger association between risk and fish consumption among post-menopausal in relation to pre-menopausal breast cancer is consistent with other findings of differences in risk associated with some factors, such as body mass, among pre- and postmenopausal women. We have found that among women with slow *NAT2* genotype, cigarette smoking was a risk factor for post-, but not premenopausal breast cancer. In light of the evidence that pre-menopausal and post-menopausal breast cancer may have different etiologies, (Velentgas and Daling, 1994; Janerich and Hoff, 1982; de Waard, 1979), this heterogeneity is plausible. The disparity in results in these analyses by menopausal status may reflect different etiologic pathways associated with menopausal status.

Our study may have been hampered by biases common to case-control studies, particularly those involving selection, dietary recall and measurement. Regarding selection bias, most case non-participation was due to physicians' refusals to allow contact with their patients (72%). Among post-menopausal women, non-participants were, on average, about 3 years older than participants. Thus, the most ill patients may not have been included, limiting generalizability. Among controls, a sample refusing interview (n = 117) was compared with a sample of participants (n = 372) in a telephone interview prior to data collection. No differences in reported meat, vegetable or fruit consumption were found. Thus, non-response among controls is unlikely to be related to dietary exposure.

For many cancers, illness may have caused changes in dietary habits, possibly influencing memory of past eating habits. Thus,

recall bias may affect observed associations between dietary intake and cancer risk, although evidence for this bias is not consistent. With breast cancer, though, the growing tumor is often asymptomatic until diagnosis; it probably does not affect appetite. Questions in our study were focused on intake in the year 2 years before the interview. Regarding measurement error, clearly, the use of a food frequency questionnaire to assess macro- and micronutrients may result in misclassification of nutrient intake. Nonetheless, it is likely that the instrument enables us to rank order subjects and identify at least strong relationships. However, this questionnaire was not designed to estimate dietary intake of heterocyclic amines, and as such, allows only use of surrogates for evaluation of associations between probable HA consumption and risk, which certainly include measurement error.

It is also becoming clear that metabolic pathways are extremely complex, involving a number of Phase I and Phase II enzymes. It is possible that effects of NAT2 may only impact on risk if CYP1A2 phenotype is also rapid, that is, rapid activation at both junctures in the metabolic pathway. This phenomenon was observed by Lang *et al.*, (1994) in a study of colon cancer, where risk was highest for those with rapid NAT2 and rapid CYP1A2 phenotypes. Lack of data on CYP1A2 may, therefore, also be related to the lack of association between meats, NAT2, and breast cancer risk.

A final caution regarding our findings is related to the size of the study group. In the overall assessment of meat and fish consumption on risk, we have adequate power to detect an effect. However, these findings may be affected by numerous sources of bias. In the analyses stratified by acetylator status, in which one would expect the bias to be non-differential and thus less of a problem, numbers are quite small. For some risk estimates, confidence intervals are wide and estimates of risk unstable. Thus, our findings must be viewed as tentative, and further studies of consumption of dietary heterocyclic amines, using a validated questionnaire for their assessment, are warranted, particularly in light of the laboratory data suggesting their association with mammary carcinogenesis.

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APPENDIX D – Risk of recurrent spontaneous abortion, cigarette smoking, and genetic polymorphisms in NAT2 and GSTM1

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Risk of Recurrent Spontaneous Abortion, Cigarette Smoking, and Genetic Polymorphisms in *NAT2* and *GSTM1*

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Maternal smoking increases the risk of spontaneous abortion. Polymorphic *N*-acetyltransferase (*NAT2*) and glutathione *S*-transferase (*GSTM1*) affect metabolism of some mutagens found in tobacco smoke. Genotypes and smoking were studied in women with at least two spontaneous abortions (N = 32) and those with at least two livebirths (N = 179). Smoking slightly increased risk (odds ratio = 1.3; 95% confidence interval = 0.6–2.9), but *NAT2* and *GSTM1* did not. *NAT2* or *GSTM1* polymorphisms did not appreciably modify smoking-related risk. (Epidemiology 1998;9:666–668)

Keywords: *N*-acetyltransferase 2, glutathione *S*-transferase M1, spontaneous abortion, genetic polymorphism, gene-environment interaction, molecular epidemiology.

Approximately 12–15% of clinically recognized pregnancies end in spontaneous abortion. Studies of recurrent (≥2) spontaneous abortion indicate a role for genetic[1,2] and possibly immunologic factors.[3] Putative environmental risk factors include cigarette smoking and coffee consumption.[1]

Tobacco smoke contains numerous compounds that might be related to spontaneous abortion, including mutagenic aromatic amines and polycyclic aromatic hydrocarbons (PAHs).[4,5] *N*-Acetyltransferase (NAT2) and glutathione *S*-transferase (GSTM1) are involved in the detoxification of aromatic amines and PAHs, respectively. Rates of metabolism of these compounds vary; three point mutations predict NAT2 slow acetylator phenotype in Caucasians,[6] and individuals homozygous for the *GSTM1* null allele are deficient for activity of that isozyme.[7] A previous study[8] of the association between *NAT2* and *GSTM1* and risk of recurrent spontaneous abortion found little evidence for an association. In this investigation, we sought to corroborate those findings. Because risk associated with metabolic variability may depend on exposure to the relevant substrate, we additionally examined possible effect modification of smoking risk by polymorphic *NAT2* and *GSTM1*.

Subjects and Methods

This analysis was based on data collected in a case-control study of breast cancer from 1986 to 1991. For these analyses, we included only women from the control group. Caucasian pre- and postmenopausal women in western New York between the ages of 40 and 85 years were enrolled (316 premenopausal and 494 postmenopausal community controls). A more detailed description of the study population has been published elsewhere.[9,10] Participants were asked to provide a blood specimen "for examination"; approximately 57% agreed, and the specimens were processed and frozen at −70°C. DNA was extracted from preserved clots, and genotyping was performed, as previously described.[10,11] *GSTM1* was evaluated for the presence or absence of alleles. Amplified polymerase chain reaction product for *NAT2* was cut with restriction enzymes to identify mutations at $C^{481}T$, $G^{590}A$, and $G^{857}A$, which have been shown to predict 90–95% of the slow acetylation phenotype in Caucasians.[12,13]

In an extensive 2-hour personal interview, women were asked about the outcome of each reported pregnancy before age 40 years. In these analyses, cases were defined as women who reported two or more spontaneous abortions, regardless of other pregnancy outcomes,

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TABLE 1. Reported Reproductive History and Smoking during Reproductive Years by *NAT2* and *GSTM1* Genotypes

	NAT2				*GSTM1*			
	Slow		Rapid		Null		Wildtype	
	Number	%	Number	%	Number	%	Number	%
≥2 livebirths								
Smoking −	56	35	52	39	41	35	46	36
Smoking +	37	23	34	26	27	23	36	26
≥2 spontaneous abortions								
Smoking −	10	6	7	5	7	6	8	6
Smoking +	8	5	7	5	5	4	6	4

and had genetic data available for *NAT2* (N = 32) or *GSTM1* (N = 26). The control groups comprised women with data on *NAT2* (N = 179) or *GSTM1* (N = 150) and at least two livebirths, that is, at least two opportunities for spontaneous abortion but no such occurrence. All women with *GSTM1* data also had *NAT2* results available.

Participants gave a detailed smoking history, including age started, times quit, and amount smoked 2, 10, and 20 years previously. To approximate smoking during the reproductive years, if a woman reported that she began smoking before her first pregnancy and did not quit for any period until after the birth of her last child, we considered her a smoker.

We used unconditional logistic regression to obtain odds ratios with 95% confidence intervals, computed from the standard error of the regression coefficient. Adjusted models did not change the point estimates appreciably; we present only unadjusted results.

Results

Table 1 shows data regarding reproductive histories and smoking habits by *NAT2* and *GSTM1* genotypes. For the most part, there were few differences by genotype. In Table 2, associations are shown between risk of recurrent spontaneous abortion and smoking during reproductive years, *NAT2* genotype, and *GSTM1* genotype. There was a slight suggestion of increased risk associated with smoking. Estimates of a smoking effect were greater in the larger dataset before exclusion of those without genetic data (data not shown). There was no meaningful association between either *NAT2* or *GSTM1* genotype and risk of recurrent spontaneous abortion.

In Table 3, we compare smokers and nonsmokers with variant *NAT2* and *GSTM1* genotypes with the putative "low-risk" gene-environment combinations. There was no indication of any interaction.

Discussion

Recall of pregnancy events, including spontaneous abortions, is reliable over time,[14] although it may be somewhat limited for losses occurring early in gestation and by the length of time since the event.[15] Misclassification of recurrent spontaneous abortion is unlikely, however, and there is no evidence to indicate that reporting would be affected by genetic or smoking status.

The number of cigarettes smoked at the most relevant time frame for the present analysis, smoking at the time of pregnancies, was not available. We constructed an index to reflect the likelihood of smoking during the reproductive years, but we cannot be certain that smoking actually occurred during the months of pregnancy, although there were no reports of quitting smoking during that time period.

Effects of caffeine exposure theoretically could be modified by NAT2 with regard to spontaneous abortion

TABLE 2. Effects of Smoking Status and Polymorphic Detoxification Genes on Recurrent Spontaneous Abortion

	Cases		Controls			
	Number	%	Number	%	Crude OR*	95% CI†
Smoking during reproductive years						
No‡	17	53	108	60	1.0	
Yes	15	47	71	40	1.34	0.63–2.86
N-Acetyltransferase 2 genotype§						
Rapid	14	44	86	48	1.0	
Slow	18	56	93	52	1.19	0.56–2.54
Glutathione S-transferase M1 genotype‖						
Wildtype	14	54	82	54	1.0	
Null	12	46	68	46	1.01	0.44–2.35

* Odds ratio.
† 95% confidence interval.
‡ Referent category.
§ Based on 32 women with two or more spontaneous abortions (cases) and 179 women with two or more livebirths (controls).
‖ Based on 26 women with two or more spontaneous abortions (cases) and 150 women with two or more livebirths (controls).

TABLE 3. Combined Effects of Smoking and Genetic Polymorphisms in Metabolizing Genes in Relation to Recurrent Spontaneous Abortion

	Cases		Controls			
	Number	%	Number	%	Crude OR*	95% CI†
N-Acetyltransferase 2 Genotype by Smoking Status during Reproductive Years‡						
Rapid						
Smoking −§	7	22	52	29	1.0	
Smoking +	7	22	34	19	1.53	0.49–4.75
Slow						
Smoking −	10	31	56	31	1.33	0.47–3.74
Smoking +	8	25	37	21	1.61	0.54–4.82
Glutathione S-transferase M1 Genotype by Smoking Status during Reproductive Years‖						
Wildtype						
Smoking −§	8	31	46	31	1.0	
Smoking +	6	23	36	24	0.96	0.31–3.01
Null						
Smoking −	7	27	41	27	0.98	0.33–2.94
Smoking +	5	19	27	18	1.06	0.32–3.59

* Odds ratio calculated by unconditional logistic regression.
† 95% confidence interval.
‡ Based on 32 women with two or more spontaneous abortions (cases) and 179 women with two or more livebirths (controls).
§ Referent category.
‖ Based on 26 women with two or more spontaneous abortions (cases) and 150 women with two or more livebirths (controls).

risk,[16] although a recent study of NAT2 phenotype found no effect modification of caffeine-related risk by genotype.[17] Information on caffeine consumption was available only for the 2 years before the interview; thus, we could not evaluate modification of caffeine use by NAT2.

Corroborating the findings of Hirvonen and colleagues,[8] we did not observe a relation between polymorphisms in *NAT2* and *GSTM1* and risk of recurrent spontaneous abortion. Nevertheless, molecular epidemiologic studies indicate that metabolic variability may confer no risk without exposure to the appropriate substrate. Although these preliminary data do not support a role for modification of smoking-related risk of recurrent spontaneous abortion by NAT2, inferences are uncertain owing to sparse data.

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APPENDIX E - *NAT1* unpublished results

Table 1
NAT1 genetic polymorphisms in premenopausal and postmenopausal cases and controls: Western New York Breast Cancer Study.

Genotype combinations	Cases (n=127) n	(%)	Controls (n=134) n	(%)	Cases (n=181) n	(%)	Controls (n=224) n	(%)
	PREMENOPAUSAL				**POSTMENOPAUSAL**			
4/3	4	(3)	2	(2)	7	(4)	7	(3)
4/4	78	(61)	79	(64)	116	(64)	130	(58)
10/3	1	(1)	1	(1)	1	(1)	0	(0)
4/10	33	(26)	29	(23)	40	(22)	59	(26)
4/11	3	(2)	8	(6)	9	(5)	10	(5)
10/10	8	(6)	4	(6)	5	(3)	14	(6)
11/10	0	(0)	1	(1)	1	(1)	4	(2)
11/11	0	(0)	0	(0)	2	(1)	0	(0)

Table 2
NAT1 Genotypes, Cigarette Smoking, and Risk of Breast Cancer: Western New York Breast Cancer Study.

NAT1 Genotype[1]	Cases (%)	Controls (%)	OR (CI) [2]	Cases (%)	Controls (%)	OR (CI) [2]
	PREMENOPAUSAL			POSTMENOPAUSAL		
All women with genetic data						
Slow	85 (67)	89 (72)	1.0	134 (74)	147 (66)	1.0
Rapid	42 (33)	35 (28)	1.3 (0.7-2.2)	47 (26)	77 (34)	0.7 (0.5-1.1)
Non-smokers						
Slow	57 (69)	64 (74)	1.0	62 (69)	73 (64)	1.0
Rapid	26 (31)	22 (26)	1.4 (0.7-2.8)	28 (31)	41 (36)	0.8 (0.5-1.6)
Smokers [3]						
Slow	28 (64)	25 (66)	1.0	72 (79)	74 (67)	1.0
Rapid	16 (36)	13 (34)	1.1 (0.4-3.0)	19 (21)	36 (33)	0.6 (0.3-1.1)

[1]Rapid genotype are women with any *NAT1*10* alleles, slow genotype are those with others (*3, *4, *11).
[2] Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, family history of breast cancer.
[3] Women were classified as smokers if they had smoked more than 1 cigarette per day for at least one year.

Table 3
NAT1 and *NAT2* Genotypes, Cigarette Smoking, and Risk of Breast Cancer: Western New York Breast Cancer Study.

NAT1 Genotype[1]	Cases (%)	Controls (%)	OR (CI) [2]	Cases (%)	Controls (%)	OR (CI) [2]
	NAT1			*NAT2*		
Non-smokers						
Rapid	62 (69)	73 (64)	1.0	41 (49)	59 (54)	1.0
Slow	28 (31)	41 (36)	1.2 (0.6-2.2)	43 (51)	50 (46)	0.9 (0.5-1.7)
Smokers [3]						
Rapid	19 (21)	36 (33)	1.0	37 (37)	50 (48)	1.0
Slow	72 (79)	74 (67)	1.8 (0.9-3.5)	64 (63)	54 (52)	1.7 (0.9-3.0)

[1]Rapid genotype are women with any *NAT1*10* alleles, slow genotype are those with others (*3, *4, *11).
[2] Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, family history of breast cancer.
[3] Women were classified as smokers if they had smoked more than 1 cigarette per day for at least one year.

Table 4
Risk of breast cancer related to combinations of *NAT1* and *NAT2* genotypes among postmenopausal women: Western New York Breast Cancer Study.

NAT1, *NAT2*	Cases n (%)	Controls n (%)	OR (CI) [1]
NON-SMOKERS			
rapid, rapid [2]	16 (23)	19 (21)	1.0
rapid, slow	7 (10)	12 (13)	0.6 (0.2-2.2)
slow, rapid	18 (26)	24 (26)	0.7 (0.3-1.9)
slow, slow	29 (41)	37 (40)	0.9 (0.4-2.2)
SMOKERS			
rapid, rapid [2]	6 (8)	18 (23)	1.0
rapid, slow	9 (12)	12 (15)	1.7 (0.5-6.3)
slow, rapid	21 (27)	19 (24)	2.6 (0.8-8.3)
slow, slow	41 (53)	31 (39)	4.0 (1.3-11.7)

[1] Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, family history of breast cancer.
[2] Reference category are women with rapid *NAT1* and rapid *NAT2* genotypes
[3] Women were classified as smokers if they had smoked more than 1 cigarette per day for at least one year.

APPENDIX F - Polymorphic alcohol dehydrogenase 3 and risk of breast cancer associated with alcohol consumption

Freudenheim JL, Ambrosone CB, Moysich KM, Vena JE, Graham S, Marshall JR, Muti P, Laughlin R, Nemoto T, Harty L, Crits A, Chan A, Shields PG. Polymorphic alcohol dehydrogenase 3 and risk of breast cancer associated with alcohol consumption. Cancer Causes and Control.

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Alcohol Dehydrogenase 3 Genotype Modification of the Association of Alcohol Consumption with Breast Cancer Risk

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Running head: ADH_3 and breast cancer risk

Abstract

Because alcohol dehydrogenase 3 (ADH_3) is rate limiting in alcohol oxidation and is polymorphic, we examined ADH_3 genotype in relation to alcohol intake and breast cancer risk. We conducted a case-control study among Caucasian women aged 40-85 with incident, pathologically confirmed breast cancer and controls, frequency-matched on age and county. Queries included alcohol intake in the past 20 years. Genomic DNA was genotyped for the exon VIII ADH polymorphism by PCR followed by restriction enzyme digestion. Computation of odds ratios (OR) and 95% confidence intervals (CI) was by unconditional logistic regression. We found increased risk among pre- (OR 2.29, 95% CI 1.24-4.26) but not postmenopausal women (OR 1.11, 95% CI 0.71-1.73) associated with ADH_3^{1-1} compared to ADH_3^{1-2} and ADH_3^{2-2} genotypes. Risk was increased for premenopausal women with the ADH_3^{1-1} genotype and alcohol intake above the median (OR 3.62, 95% CI 1.50-8.78) compared to lighter drinkers with the ADH_3^{2-2} or ADH_3^{1-2} genotypes. ORs were close to the null for premenopausal women in other drinking and genotype groups and for postmenopausal women categorized by genotype and alcohol consumption. Among premenopausal women, there may be a group more genetically susceptible to an alcohol consumption effect on breast cancer risk.

Key words: alcohol, alcohol dehydrogenase, breast neoplasms, epidemiology, genetic polymorphisms

Introduction

While there is evidence that alcohol consumption may increase the risk of breast cancer (1-3), the mechanism of action is not well understood. It is possible that genetic differences in the metabolism of alcohol may alter the relation of alcohol exposure to breast cancer. Evaluation of heterogeneous groups may mask susceptible subgroups and impair estimation of effects. In this study, we evaluated genetic variation in alcohol dehydrogenase, a key enzyme in alcohol metabolism, as a modifying factor in the relation between alcohol intake and breast cancer risk.

Alcohol dehydrogenase (ADH) catalyzes the oxidation of ethanol to acetaldehyde and plays a rate-limiting role in the metabolic pathway for most human ethanol oxidation. Dimeric class I ADH enzymes are comprised of subunits encoded by genes designated as ADH_1, ADH_2, and ADH_3. Genetic variants with altered kinetic properties have been identified at the ADH_2 and ADH_3 loci (4). The aldehyde dehydrogenase family of enzymes (ALDH) is also involved in alcohol metabolism and variant alleles with altered kinetic activities have been identified in the $ALDH_2$ gene (4). Polymorphisms in ADH_2 and $ALDH_2$ are rare in Caucasian populations (4-6). In one study of the ADH_3 gene, approximately 58%, 91% and 88% of European whites, Asians and Africans, respectively had the ADH_3^1 allele (5). For this study of Caucasians, we examined effects of the ADH_3 polymorphism. There is evidence that this variant has functional importance. *In vitro,* there is more than two-fold difference in V_{max} between the ADH_3 genotypes (4), with the ADH_3^1 allele coding for the more rapid form of the enzyme.

While there are, to our knowledge, no reports on the association of ADH_3 in relation to breast cancer, there have been reports of an association of the ADH_3^{1-1}

genotype with increased risk of cancer of the oral cavity and pharynx (7,8) and of hepatic cirrhosis and chronic pancreatitis (6). We report here on the results of a case-control study of breast cancer risk with an examination of associations of alcohol consumption stratified by ADH_3 genotype.

Materials and Methods

We conducted a case-control study of breast cancer in pre- and postmenopausal women in western New York State. All participants provided written informed consent; procedures for protection of human subjects in this study were approved by the Human Subjects Review Board of the State University of New York at Buffalo School of Medicine and Biomedical Sciences and of each of the participating hospitals. The women in the study were between the ages of 40 and 85, residents of Erie and Niagara counties, alert, able to speak English and in sufficiently good health to be interviewed; all were Caucasian. Women were considered to be premenopausal if they were currently menstruating or, if they were not menstruating because of a hysterectomy or other medical intervention, if they had at least one of their ovaries and were less than age 50. All other women were considered to be postmenopausal.

Women with incident, primary, histologically-confirmed breast cancer were identified from pathology records of all the major hospitals in the two counties; case ascertainment was conducted in the period beginning November, 1986 and ending October, 1989 for postmenopausal cases, and ending April, 1991 for premenopausal cases. The physician of each woman identified with breast cancer was contacted to obtain consent to allow us to invite the woman for an interview. Of eligible cases, 66% of premenopausal and 54% of postmenopausal cases were interviewed. Physician refusal to allow us to contact their patients accounted for most of the lack of participation, 74% and 71% of nonparticipation for

pre- and postmenopausal women, respectively. Interviews were conducted, on average, two months after diagnosis; no interviews were conducted more than one year after diagnosis.

Controls were frequency-matched to cases on age and county. The listing of licensed New York State drivers was used for random selection of women under age 65; women age 65 and over were randomly selected from the listing of the Health Care Finance Administration. Interviewed were 62% and 44%, respectively, of the eligible pre- and postmenopausal controls. Because controls under age 65 were licensed drivers, we asked the cases under 65 if they had driver's licenses. Nine did not hold a driver's license. Compared to cases with licenses, women without licenses were slightly less educated and slightly, though not significantly older. All are included in these analyses. For a subset of participating controls and those refusing to participate, we conducted a very brief phone interview querying usual frequency of consumption of several foods. These participants and non-participants did not differ in reported intake of vegetables, fruits, meat or coffee. Non-participants were somewhat more likely to smoke. Information was not collected on alcohol intake in this comparison of participants and non-participants (9,10).

Interviews Interviews were conducted in the participants' homes by trained interviewers. The interview lasted, on average, two hours. Details of the interview have been described elsewhere (9-11). Included in the interview were questions regarding usual diet in the year two years before interview, reproductive history, medical history, family history of cancer, smoking history (pack-years) and other breast cancer risk factors. Body mass index (BMI) was calculated from reported height and weight, as weight (kg)/height^2(m^2). Family history of breast cancer was defined as having at least one first-degree relative (mother, sister, daughter) with breast cancer.

Questions regarding alcohol intake included queries of the usual frequency of intake and number of drinks per occasion for wine, beer and hard liquor during the year two years ago, 10 years ago, 20 years ago and at age 16. Total alcohol intake was calculated as the sum of the reported number of drinks of beer, wine and hard liquor under the assumption that the alcohol content for one glass of beer or wine or one shot of hard liquor were approximately the same. An index of usual alcohol consumption in the last 20 years was estimated as a weighted sum of the reported intakes for two years ago, 10 years ago and 20 years ago.

At the end of the interview, participants were asked to provide a blood sample following an additional informed consent. About 45% of premenopausal and 63% of postmenopausal participants agreed to give a blood sample.

Molecular Genetic Analyses All analyses were conducted at the Laboratory for Human Carcinogenesis at the National Cancer Institute. DNA was extracted from blood clots (11). As previously described (7), a 145 bp fragment including the Exon VIII polymorphism was amplified by the polymerase chain reaction (PCR) using a modification of the method of Groppi et al. (12). The highly homologous ADH_1 and ADH_2 genes were digested with the NlaIII restriction enzyme prior to the PCR. An aliquot of this digestion mixture was then subjected to PCR and subsequent SspI enzymatic digestion to reveal the ADH_3 genotype (i.e., ADH_3^{1-1}, ADH_3^{1-2} , or ADH_3^{2-2}). Every 14 samples contained a positive and negative control. The results were scored separately by two authors independently who were blinded to all identifying data including subjects' case-control status. Twenty percent of samples were repeated for quality control. In the adjusted analyses, NAT2 genotype was examined as a potential adjusting variable; methodology for the NAT2 analyses have been described previously (11).

The final sample for this report included 134 premenopausal cases and 126 premenopausal controls, 181 postmenopausal cases and 230 postmenopausal controls, those women whom we interviewed and whose ADH_3 genotype could be determined. Because we did not get blood samples from all participants who completed the interview nor were we able to successfully determine the ADH_3 polymorphism on all blood samples, we compared the characteristics of those included in this report with the entire group included in the case-control study; comparisons of means were made using the student's t-test. Those with and without ADH_3 data were largely similar with a few exceptions. Differences ($p<0.05$) among premenopausal women were that those with data tended to be older, have higher parity and to drink less beer than those without. Among postmenopausal women, the only characteristic that was significantly different was age; those with ADH_3 data were older.

Statistical Analysis Because there are indications that there are differences in the risk factors for pre- and postmenopausal breast cancer (13) and in particular because there may be differences in the effect of alcohol intake depending on menopausal status (1), analyses were stratified by menopausal status. For potential confounding factors, means and standard deviations for groups defined by ADH_3 genotype and by case-control status were compared by one-way analysis of variance, with a two-tailed test of significance; values for categorical data were compared using the chi-square test (14). Odds ratios (OR) and 95% confidence intervals (CI) were calculated using unconditional logistic regression (15). For analyses of categorical data, odds ratios were calculated relative to the indicated referent category. Cutoffs for categories of alcohol intake were at the median level of intake for controls. Because of limitations in sample size, it was not possible to examine groups with more narrowly defined alcohol intakes. Adjusted analyses included control for age, education, family history of breast cancer, reported history of benign breast disease, BMI, parity, age at

first birth, age at menarche, fruit and vegetable intake, duration of lactation and, for postmenopausal women, age at menopause. Most of these factors were examined for confounding effects because they have been found to be associated with risk of breast cancer. We also examined possible confounding by smoking history, NAT2 status and smoking by NAT2 interaction because we had previously found these to associated with risk in this population (11). Odds ratios for the ADH_3 genotypes were calculated and then odds ratios for alcohol intake both without and with stratification on ADH_3 genotype were calculated. Because of issues regarding differential recall for cases and controls in case-control studies, we also examined a case-case analysis in relation to alcohol dehydrogenase status; alcohol intake was regressed on ADH genotype among the cases with the ADH_3^{2-2} and ADH_3^{1-2} groups combined as the referent with comparison to ADH_3^{1-1} (16,17).

Results

For all analyses, the cutoff between the lower and higher groups of drinkers was at the median for controls, 6.5 and 4.4 drinks per month on average over the last 20 years, for the pre- and postmenopausal women respectively. The associations between reported alcohol consumption in the last 20 years and risk of breast cancer in this sample of individuals with available genetic data are shown in Table 1. For both pre- and postmenopausal women, confidence intervals included the null. For the premenopausal women, there was a suggestion of increased risk among heavier drinkers. Similar results were obtained when all the data including participants who did not provide a blood sample were analyzed. We also examined risk associated with alcohol consumption separately for the reports of alcohol consumption two years ago, ten years ago and twenty years ago. Odds ratios for these periods were similar to those

shown for the combined index; confidence intervals overlapped for all three periods for pre- and postmenopausal women.

In Table 2, breast cancer risk factors are shown for cases and controls grouped by genotype. In general, characteristics of the three genotype groups within the cases and the controls were similar. For alcohol intake, values shown are for all subjects combined, including non-drinkers. For the premenopausal women, 4% of cases and 6% of controls were non-drinkers; for postmenopausal women, non-drinkers constituted 13% and 11% of cases and controls, respectively. The percentage of non-drinkers did not differ by genotype in any of the groups defined by case-control and menopausal status. In one-way analysis of variance, the reported alcohol intakes of were not different by genotype for either the pre- or postmenopausal controls. For premenopausal cases with the ADH_3^{2-2} genotype, reported alcohol intakes were significantly higher than those with the ADH_3^{1-2} ($p<0.05$), but not the ADH_3^{1-1} genotype. There were also some differences in smoking history between the homozygotes and the heterozygotes among the premenopausal women. Among postmenopausal women, alcohol consumption and smoking did not differ for the different groups; there was a difference by genotype for education among the cases.

In Table 3, risk of breast cancer associated with ADH_3 genotype is shown. There was an increase in risk for the premenopausal women associated with the ADH_3^{1-1} genotype; the confidence interval included the null value (adjusted OR 1.96; 95% CI 0.85-4.59). There was little evidence of an association of genotype with risk for postmenopausal women. Odds ratios estimated without adjusting for alcohol intake were similar to those shown here. Addition of smoking, NAT2 and an interaction term of NAT2 and smoking did not appreciably change the estimates.

We also examined risk of breast cancer associated with the ADH_3^{1-1} genotype when the referent was the ADH_3^{2-2} and ADH_3^{1-2} genotype groups combined. For premenopausal women, the odds ratio was 2.29 (95% CI, 1.24-4.26); for postmenopausal women the odds ratio was 1.11(95% CI, 0.71-1.73) (data not shown).

In Table 4, odds ratios for alcohol intake by ADH_3 genotype are shown. The referent was women with lower intake of alcohol and either the ADH_3^{2-2} or ADH_3^{1-2} genotype. (We also analyzed these data with ADH_3^{2-2} alone as the referent. The results were similar to those shown here. However, the findings were less stable because the sample size in the reference group was small and confidence intervals were wider.) Among the premenopausal women, odds ratios were generally close to the null and confidence intervals included the null for all categories with one exception. Among women who drank more than the median intake and who had the ADH_3^{1-1} genotype, the odds ratio was 3.62 with 95% confidence interval 1.50-8.78. It appeared that the effect associated with both the ADH_3^{1-1} genotype and higher alcohol consumption was more than additive; however, the multiplicative interaction term in a logistic regression was not significantly different from the null (p=0.16). The estimates of risk in Table 4 were essentially unchanged when smoking, NAT2 and smokingxNAT2 were included in the model. We also examined risk associated with alcohol within the group of women with the ADH_3^{1-1} genotype. With lighter drinkers as the referent, the adjusted odds ratio for drinking more than the median of alcohol was 3.86, 95% confidence interval 1.34-10.06 (data not shown). Additionally, we repeated this latter analysis, changing the cutpoint for the low and high drinkers so that there was an even distribution within the premenopausal controls with the ADH_3^{1-1} genotype. The results were essentially the same (OR 3.88, 95% confidence interval 1.38-10.88).

Among postmenopausal women, there was no evidence of an association of alcohol intake and risk when modification by ADH_3 was taken into account. Because of reports that an increased risk associated with alcohol consumption among postmenopausal women may be restricted to those who have used estrogen replacement therapy (ERT) (18,19), we also looked at the odds ratios among women who had ever used ERT. Among the heavier drinkers with the ADH_3^{1-1} genotype compared to lighter drinkers with the other ADH_3 genotypes for women who had ever used ERT, the adjusted OR was1.21 and the 95% CI 0.84-1.65; for those who had never used ERT, the OR was 1.02 and the 95% CI 0.94-1.64. Sample size was quite small for the cells in these analyses; there were only 10 cases and nine controls with the ADH_3^{1-1} genotype that had ever used ERT. All of these analyses were based on reports of alcohol consumption in the last 20 years. We had also queried regarding alcohol intake at age 16. The number of drinkers at that age was too small to estimate whether there was a modifying effect of ADH_3 genotype.

In a case-case analysis, we examined risk associated with the ADH_3^{1-1} genotype compared to the combined ADH_3^{1-2} and ADH_3^{2-2} groups. As for the case-control analyses, there was evidence of some increase in risk associated with the ADH_3^{1-1} genotype for pre- but not postmenopausal women. For premenopausal women, risk was more than doubled for women drinking more than the median compared to lighter drinkers (adjusted OR 2.46, 95% CI 1.08-8.36). For the postmenopausal women the adjusted OR was 1.02 and the 95% CI was 0.41-2.10 (data not shown).

Discussion

This study of women in western New York provides evidence that the association of alcohol consumption with breast cancer risk may differ depending on genotype.

Among premenopausal women, we found an increase in risk of more than three and a half fold for drinkers above the median with the ADH_3^{1-1} genotype. We did not find an increase in risk for heavier drinkers with the other genotypes. Further, we did not find any indication in this population of generally light drinkers of a modifying effect of ADH_3 genotype among postmenopausal women. To our knowledge, this is the first study of the relation of the ADH_3 polymorphism with alcohol and breast cancer risk. As noted above, there is some indication of an increase in risk of other alcohol-related diseases among individuals with the ADH_3^{1-1} genotype, including reports of a 2.5 to 6-fold increase in risk of oral and pharyngeal cancer (7,8). There are a considerable number of studies that indicate that alcohol is related to increased risk of breast cancer (1-3). Some (20-24), but not all (1) studies find risk associated with alcohol intake particularly among premenopausal women.

This modification of the association between alcohol consumption and risk of breast cancer by ADH_3 genotype may provide some indication as to the mechanism of effect of alcohol exposure. Alcohol metabolism in humans is regulated primarily by the ADH system of enzymes. There is considerable evidence that acetaldehyde, the product of alcohol dehydrogenase oxidation of alcohol, has carcinogenic properties (25). Acetaldehyde is mutagenic and carcinogenic in experimental animals. In short term cell culture assays, including assays of human cells, acetaldehyde but not ethanol is mutagenic (26,27). *In vitro,* acetaldehyde effects include DNA adducts (28,29), DNA crosslinks and DNA-protein crosslinks (30,31) and inhibition of DNA repair (30). The International Agency for Research on Cancer (IARC) has indicated that the evidence regarding acetaldehyde is sufficient for it to be designated as a carcinogen in experimental animals (32). *In vitro,* the V_{max} for ADH_3^{1-1} is more than two-fold greater

than for ADH_3^{2-2} (4) and may therefore contribute to increased exposure to acetaldehyde. It should be noted, however, that in one study in Caucasians, no difference was found in blood ethanol levels for different ADH_3 genotypes (33). There is evidence of measurable levels of circulating acetaldehyde in premenopausal women after consumption of moderate amounts of alcohol during the high estrogen phases of the menstrual cycle (34,35). There is also evidence of acetaldehyde excretion in human milk (36); however, the determinations in milk were not made in conjunction with alcohol consumption. ADH_3 expression is greatest in the liver; however there is evidence of ADH_3 activity in other organs (37-42) with an indication of expression particularly in epithelial cells (41).

Another possible mechanism involving ADH and alcohol is with regard to steroid hormone metabolism. There is strong evidence that estrogen exposure is an important contributor to breast cancer risk (43). Alcohol consumption appears to affect estrogen levels; there is evidence that both acute (44-46) and chronic (47-49) alcohol consumption lead to increased estrogen levels in premenopausal women and in postmenopausal women who take exogenous estrogen. ADH_3 also is involved in steroid hormone metabolism and is inhibited by testosterone (50,51). If the association of ADH_3 with risk is the result of an interaction with steroid hormones, that mechanism might explain why we saw an association with risk only among the premenopausal women. Given the toxic effects of acetaldehyde, the apparent likelihood of exposure to breast tissue of acetaldehyde and the interactions of alcohol, ADH_3 and estrogens, these mechanisms together may explain, at least in part, an association of alcohol consumption with breast cancer risk. Of course, there are other possible mechanisms

that may also explain the association of alcohol with breast cancer risk that also need to be considered.

In interpretation of these findings, several potential sources of error need to be considered. In this study, all measures of alcohol intake were by self-report and measurement error is of concern. However, there is some evidence that reliability of recall of intake of alcohol in the past five to ten years is relatively good (52,53), although current drinking practices may bias recall of intake (52). In data such as ours, there is also the concern of recall bias, that women with recently diagnosed breast cancer may report their previous alcohol intake differently than the healthy controls do. In one study, this potential source of bias accounted for only a small reduction in the relative risk estimate with bias toward the null (54). As for the measure of ADH_3 status, there may also be some misclassification of the clinically significant ethanol oxidation phenotype. Methodologically, however, laboratory personnel were blinded to case-control status; error with regard to ADH_3 status would be non-differential and would contribute to an attenuation of the odds ratio estimate (55).

In terms of the selection of the sample, while every effort was made to include a population-based sample in this study, there were several sources of non-participation. For the cases, the largest source of non-participation was the refusal of physicians to allow us to contact the women. It may be that this lack of inclusion reflects physician rather than patient characteristics, but we could not verify whether or not this was true. Among the controls, we do have some evidence that at least for dietary intake, there were no differences among participants and those who did not participate (9,10). There may have been differences in alcohol intake of those refusing to participate; in particular, it is possible that the heaviest drinkers in the population were

underrepresented. For both cases and controls, there is no reason to believe that participation would be related to ADH_3 polymorphism; the frequency of the ADH_3^1 and ADH_3^2 alleles measured in this population (59% and 41%, respectively among the controls) were similar to those reported by others (4,5,56). ADH_3 would be unlikely to affect alcohol consumption; studies of ADH_3 in Caucasians have not shown there to be differences in risk of alcoholism associated with the ADH_3 genotype (6,56). We did not find any difference in alcohol intake by ADH_3 polymorphism among the controls. Among premenopausal cases, reported alcohol intake was lower for the ADH_3^{1-2} genotype than for the ADH_3^{2-2}; this finding does not make biological sense in the context of the other groups where there were no differences. It may be that this apparent difference is the result of chance. There were no differences in intake for the other comparisons within in the cases, for the controls or for the postmenopausal cases or controls.

Possible confounding is of concern as well. Known risk factors for breast cancer were examined as potential confounders. The possibility remains that there were other correlated exposures that may explain the observed associations. In particular, it could be that there is confounding by genetic admixture even within this group of Caucasians. In addition, rather than the observed associations being an effect of ADH_3, it could be that the observed association is the result of linkage disequilibrium of ADH_3 with another gene causally related to breast cancer.

Finally, there is also the possibility that these findings were the result of chance. Given the small samples in some of the cells of analysis and given the issues of potential bias, these results necessarily need to be considered as preliminary and await confirmation by other, larger epidemiologic studies. Because of the restriction by sample size, we were only able to categorize participants into two levels of drinking. The group

of heavier drinkers necessarily included women whose alcohol consumption was in fact rather low. Additionally, the group of lighter drinkers included both non-drinkers and those who drink less frequently. With larger sample size and the ability to examine risk in groups that are more narrowly defined by alcohol consumption, it would have been possible to elucidate the association of drinking, genotype and risk.

Our data suggest that genetic differences in alcohol metabolism by ADH_3 should be considered as possible modifiers of the association between alcohol intake and breast cancer. In other studies, consideration of genetic variation in ADH_2 and ALDH, which we were not able to study, is warranted. Further, given the small number of individuals in some of the genotype-alcohol categories, chance may explain the findings; replication in other populations would be of importance. Our findings of an apparent modification of effect by ADH_3 genotype, if confirmed in other studies, would shed some light on the possible mechanism of an alcohol consumption effect on breast cancer and indicate a high-risk group for an alcohol effect.

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Table 1

Alcohol Consumption in the Last 20 Years and Risk of Breast Cancer, Western New York, 1987-1991 (Subgroup of Women with Alcohol Dehydrogenase 3 (ADH_3) Genotype Measured)

Alcohol*	Cases	Controls	Crude OR	Adjusted OR**	95% CI
Premenopausal					
Lower	54	63	1.0	1.0	
Higher	80	63	1.5	1.6	(0.9-2.6)
Total	134	126			
Postmenopausal					
Lower	93	113	1.0	1.0	
Higher	88	117	0.9	0.9	(0.6-1.5)
Total	181	230			

*Cutoffs for lower and higher groups of drinkers were the medians of the reported average consumption over the last 20 years; the cutoff was 6.5 and 4.4 drinks per month, for pre- and postmenopausal women, respectively.

**OR = odds ratio; CI = confidence interval. Adjusted for age, education, family history of breast cancer, history of benign breast disease, BMI, parity, age at first birth, age at menarche, fruit and vegetable intake, alcohol intake in the last 20 years, lifetime duration of lactation, and age at menopause (postmenopausal women only).

Table 2
Characteristics of Study Sample by Case and Control Status and Alcohol Dehydrogenase 3 (ADH_3) Genotype

	Premenopausal Women					
	Cases			Controls		
Characteristic*	ADH_3^{1-1}	ADH_3^{1-2}	ADH_3^{2-2}	ADH_3^{1-1}	ADH_3^{1-2}	ADH_3^{2-2}
Age (yrs)	46.2 (4.6)	46.8 (4.0)	45.0 (3.2)	46.2 (3.2)	46.9 (3.7)	47.5 (4.5)
Education (yrs)	13.6 (2.5)	14.0 (3.2)	14.1 (2.5)	14.1 (2.4)	13.6 (2.8)	14.2 (2.6)
Age at menarche (yrs)	12.6 (1.8)	12.4 (1.6)	12.5 (1.2)	13.1 (1.9)	12.9 (1.7)	13.3 (1.7)
Body Mass Index**	24.2 (5.2)	25.2 (6.0)	24.7 (4.8)	25.2 (4.6)	25.6 (4.1)	26.6 (6.0)
History of benign breast disease (% of cases or controls)	22	20	7	15	13	9
Family history of breast cancer (% of cases or controls)	8[a]	8[b]	2[a,b]	0.8	0.4	0.8
Total Alcohol** (drinks/month)	16.8 (20.2)	9.7[c] (11.3)	20.2[c] (24.4)	14.2 (28.0)	13.4 (18.1)	12.5 (12.8)
Duration lactation (months)	4.0 (8.6)	2.1 (4.3)	2.7 (5.0)	8.0 (14.1)	5.2 (12.1)	6.2 (13.1)
Parity	2.5 (1.6)	2.1 (1.5)	1.9 (1.3)	2.4 (1.6)	2.8 (1.8)	3.0 (1.7)
Age at first birth (yrs)	24.0 (4.4)	24.0 (4.8)	23.8 (5.2)	22.8 (4.0)	21.9 (4.1)	21.8 (4.0)
Vegetable intake** (g/day)	459(220)	395 (180)	419 (175)	462 (190)	473 (201)	450 (155)
Fruit intake** (g/day)	239 (133)	210 (141)	170 (125)	272(170)	245 (149)	216 (112)
Smoking (pack-yrs)	11.7[d] (16.4)	5.8[d] (10.1)	12.6 (14.4)	5.7[e] (11.4)	11.7[e,f] (16.6)	4.8[f] (8.9)
N	63	50	21	42	60	24

Table 2 continued
Postmenopausal Women

Characteristic*	Cases ADH_3^{1-1}	Cases ADH_3^{1-2}	Cases ADH_3^{2-2}	Controls ADH_3^{1-1}	Controls ADH_3^{1-2}	Controls ADH_3^{2-2}
Age (yrs)	64.9 (6.4)	63.6 (7.8)	61.9 (7.5)	63.4 (7.7)	63.1 (7.2)	61.6 (6.7)
Education (yrs)	12.2[e] (2.6)	12.3 (2.9)	13.4[e] (3.2)	12.3 (2.6)	12.0 (2.3)	12.7 (2.5)
Age at menarche (yrs)	13.0 (1.8)	13.0 (1.6)	12.6 (1.4)	12.7 (1.7)	13.1 (1.6)	12.6 (1.3)
Age at menopause (yrs)	47.8 (5.3)	47.6 (6.1)	46.8 (5.5)	46.2 (6.0)	47.6 (5.3)	47.0 (6.0)
Body Mass Index**	25.7 (5.3)	26.0 (5.0)	25.6 (3.6)	25.2 (4.2)	25.7 (5.4)	25.4 (4.7)
History of benign breast disease (% of cases or controls)	6	12	2	8	8	3
Family history of breast cancer (%)	6	6	5	3	6	1
Total Alcohol** (drinks/month)	11.8 (21.9)	17.1 (31.4)	17.7 (29.8)	10.6 (16.6)	15.9 (25.2)	12.6 (15.2)
Duration of lactation (months)	3.5 (5.7)	4.6 (11.0)	4.0 (8.0)	6.2 (10.7)	4.2 (9.0)	5.1 (10.0)
ERT (% ever used of cases or controls)	9	11	6	10	15	9
Parity	3.1 (2.0)	2.7 (2.0)	3.2 (2.8)	2.8 (2.2)	3.1 (2.0)	2.9 (1.8)
Age at first birth (yrs)	24.8 (5.0)	24.1 (4.9)	23.4 (5.2)	23.3 (4.6)	23.5 (4.6)	23.3 (3.8)
Vegetable intake** (g/day)	451 (201)	406 (175)	417 (207)	458 (237)	456 (227)	484 (334)
Fruit intake** (day)	298 (175)	254 (175)	287 (177)	306 (186)	282 (172)	308 (218)
Smoking (pack-yrs)	14.6 (21.0)	17.4 (21.3)	16.9 (29.1)	12.9 (16.5)	13.8 (19.3)	13.0 (23.0)
N	64	89	28	81	114	35

Table 2 continued

*Values shown are mean (SD) except for history of benign breast disease and family history of breast cancer which are percent with positive history. Two-sided comparisons of means between the ADH_3 groups within cases or controls were computed by ANOVA; comparisons of categories were with the chi-square test. Those with the same letter are significantly different, $p<0.05$.

**Body mass index (kg/m^2) calculated from reported height and weight two years before the interview. Alcohol values are average drinks per month during the last 20 years, calculated from the weighted sum of reported consumption two, 10 and 20 years ago; values include non-drinkers. Vegetable and fruit intake is reported intake in the year two years before the interview.

Table 3

Alcohol Dehydrogenase 3 Polymorphisms and Risk of Breast Cancer, Western New York, 1987-1991

Premenopausal ADH_3	Cases	Controls	Crude OR	Adjusted OR*	95% CI*
2-2	21	24	1.0	1.0	
1-2	50	60	1.0	0.8	(0.4-1.8)
1-1	63	42	1.7	2.0	(0.8-4.6)
Total	134	126			
Postmenopausal					
2-2	28	35	1.0	1.0	
1-2	89	114	1.0	1.1	(0.6-2.1)
1-1	64	81	1.0	1.2	(0.6-2.3)
Total	181	230			

*OR = odds ratio; CI = confidence interval. Adjusted for age, education, alcohol intake, family history of breast cancer, history of benign breast disease, BMI, parity, age at first birth, age at menarche, fruit and vegetable intake, lifetime duration of lactation and age at menopause (postmenopausal women only).

Table 4

Lifetime Alcohol Consumption by ADH_3 Genotype and Risk of Breast Cancer, Western New York, 1987-1991

Alcohol*	Cases	Controls	Crude OR	Adjusted OR**	95% CI*
Premenopausal					
ADH_3^{2-2} + ADH_3^{1-2}					
Low	33	38	1.0	1.0	
High	38	46	1.0	0.8	(0.4-1.7)
ADH_3^{1-1}					
Lower	21	25	1.0	1.0	(0.4-2.5)
Higher	42	17	2.8	3.6	(1.5-8.8)
Postmenopausal					
ADH_3^{2-2} + ADH_3^{1-2}					
Lower	60	69	1.0	1.0	
Higher	57	80	0.8	0.8	(0.47-1.38)
ADH_3^{1-1}					
Lower	34	46	0.8	0.9	(0.5-1.6)
Higher	30	35	1.0	1.2	(1.1-2.2)

*Cutoffs for lower and higher groups of drinkers were the medians of the reported average consumption over the last 20 years; the cutoff was 6.5 and 4.4 drinks per month, for pre- and postmenopausal women, respectively.

**OR = odds ratio; CI = confidence interval. Adjusted for age, education, family history of breast cancer, history of benign breast disease, BMI, parity, age at first birth, age at menarche, fruit and vegetable intake, duration of lactation and age at menopause (postmenopausal women only).

APPENDIX G – Apolipoprotein E genetic polymorphism, serum lipoproteins and breast cancer risk

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Apolipoprotein E genetic polymorphism, serum lipoproteins, and breast cancer risk.

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Abbreviated title: apoE genotype and breast cancer

Key Words: breast neoplasms, molecular epidemiology, apolipoprotein E allelic frequency, dietary fat, serum lipoproteins

List of Abbreviations:

apoE - apolipoprotein E

BMI - body mass index

CI - confidence interval

HDL - high density lipoprotein

LDL - low density lipoprotein

OR - odds ratio

PCR - polymerase chain reaction

RFLP - restriction fragment-length polymorphism

SD - standard deviation

Abstract

Apolipoprotein E (apoE) is a polymorphic gene involved in lipid metabolism with three common variant alleles (ε2, ε3, and ε4). The ε4 allele has been associated with elevated levels of cholesterol, as well as greater risk of coronary heart disease and Alzheimer's disease. In this case-control study we examined whether apoE genotype affected the association between serum lipids and breast cancer risk. In a subset of a study in western New York, 260 women with incident, primary breast cancer and 332 community controls were interviewed and provided a blood sample. PCR-RFLP analyses of the apoE polymorphism were performed. Participants were classified as apoE 2 (ε2, ε2 or ε2, ε3), apoE 3 (ε3, ε3), or apoE 4 (ε4, ε4 or ε4, ε3). Unconditional logistic regression was used to compute adjusted odds ratios (ORs) and 95% confidence intervals (CI). Compared to women with the apoE 3 genotype, there were no associations with risk for women with the apoE 2 (OR=1.0; 95% CI 0.91-1.64) or apoE 4 genotype (OR=0.97; 95% CI 0.63-1.54). Higher serum levels of total cholesterol, HDL cholesterol, and LDL cholesterol were not associated with risk, either in the total sample or among subgroups of women defined by apoE genotype. Women with the highest serum triglyceride levels had an increase in risk (OR=1.63; 95% CI 1.03-2.59) compared to women with the lowest levels. This effect was not apparent among women with the apoE 2 or apoE 3 genotype, but much stronger among women with the apoE 4 genotype (OR=4.69; 95% CI 1.49-14.7). These data suggest that the apoE 4 genotype may modify the association between serum triglycerides and breast cancer risk.

Apolipoprotein E (apoE) is a 299-amino acid glycoprotein that is synthesized by most tissues throughout the body [1]. apoE plays an important role in lipid metabolism by mediating the binding of lipoprotein particles to the low density lipoprotein (LDL) receptor and the apoE receptor [2]. The structural gene for apoE is polymorphic. There are three common isoforms, E2, E3, and E4, that are coded by the alleles ε2, ε3, and ε4, respectively, at a single locus on the long arm of chromosome 19. The apoE2 protein differs from the wild type protein, apoE3, by a single amino acid change resulting in minimal receptor binding activity and reduced clearance of chylomicron remnants [3]. ApoE4 differs from apoE3 in that a different amino acid substitution results in faster chylomicron clearance [3]. In general, compared to individuals with the ε3 allele, levels of total and LDL cholesterol tend to be lower for those with the ε2 allele and higher for those with the ε4. The ε4 allele has been associated with increased risk for coronary heart disease [4, 5] and Alzheimer's disease [6] and has been found to be underrepresented in elderly populations [7], including elderly coronary heart disease patients [5] and elderly smokers [8]. In contrast, the frequency of the ε4 allele was lower among patients with proximal tumors of the colon than among healthy individuals [9].

Dietary fat intake has long been hypothesized to be associated with breast cancer risk [10] based on animal studies [11], ecologic studies [12, 13], and studies of migrants from areas with low fat intake to those with high fat intake [14]. However, many analytic epidemiologic studies have not shown an effect of fat, including the results of a pooled analysis of seven cohort studies [15]. Recently, it has been suggested that diet in childhood and at the time of puberty may be of importance [16]. Evidence from animal studies suggests that only fat intake before the first pregnancy affects risk [17]. Studies of cholesterol intake and risk also have generally not shown an association [18, 19]. It has been suggested that the

failure to identify an association of fat intake with breast cancer in these studies my be because inter-individual differences in fat consumption within populations may be insufficient to be detected with epidemiologic methods [20] and because of measurement error inherent in dietary questionnaires [21]. It may also be that the measurement of recent diet is the wrong exposure and that diet earlier in life is more important. Also, the inter-country differences may be related to other, correlated differences in dietary intake or other exposures.

Blood levels of lipoproteins have also been investigated in relation to breast cancer etiology as a potential mediating effect of dietary fat on risk and as an independent risk factor. The associations between serum or plasma total cholesterol, high density lipoprotein (HDL) cholesterol, LDL cholesterol, and triglycerides have been widely studied, but results from these investigations are inconsistent. Some studies reported no adverse effect of lipoproteins [22-26], others reported excess risk associated with elevated total cholesterol levels [27-28] and triglycerides [29-33], or inverse associations with total cholesterol [28, 34] or HDL cholesterol levels [30, 31, 35].

Because apoE might modify the association between dietary intake and blood lipid levels, in this population based case-control study, we examined the association between apoE allelic frequency, serum lipoproteins, and breast cancer risk.

Materials and Methods

Study Population This research utilized a subset of data collected as part of a case-control study (1986-1991) of 1550 Caucasian women in Western New York. The protocol for the study was reviewed by the Institutional Review Board of the State University of New York at Buffalo, and of participating hospitals. Informed consent was received from all participants. Women diagnosed with incident, primary, histologically confirmed breast cancer (n=740) were frequency-matched by age

and county of residence with controls (n=810), randomly selected from the New York State Motor Vehicle lists (<65 years) and the Health Care Finance Administration rolls (≥65 years).

Interview Interviews were conducted in the participants' homes by trained interviewers. The interview lasted, on average, two hours. Details of the interview have been described elsewhere [36]. Included in the interview were questions regarding usual diet in the year two years before interview, reproductive history, medical history, family history of cancer, smoking history and other breast cancer risk factors. Body mass index (BMI) was calculated from reported height and weight, weight (kg)/height^2 (m^2). Family history of breast cancer was defined as having at least one first-degree relative (mother, sister, daughter) with breast cancer. At the end of the interview, participants were asked to provide a blood sample following an additional informed consent. Of women who provided usable interviews, approximately 55% agreed to donate a blood sample. Among women with breast cancer, the average time between diagnosis and blood draw was about two months. The final sample included 260 breast cancer cases and 332 community controls, who were interviewed, provided a fasting blood sample, and whose apoE genotype could be determined.

Because we did not have genetic data on all participants, we compared characteristics of controls included in this research with those for whom apoE data was not available. Cases and controls with and without apoE data were similar with respect to years of education, dietary fat intake, serum lipids, cigarette smoking, body mass index, age at menarche, and age at menopause (postmenopausal women). Cases who were included in these analyses tended to be leaner and to consume less alcohol than those for whom apoE was not available. Controls with apoE determinations tended to be slightly older, have more children, have lactated for longer duration, and to consume less alcohol than controls without apoE data.

Laboratory Analyses DNA was extracted from blood clots obtained following centrifugation and removal of serum, which had been stored at –70° C [37]. PCR conditions were based on reactions designed specifically for the APO E gene by Hixson et al [38]. Briefly, genomic DNA (≤ 30ng) was amplified using 20 pmol of primers (5'-ACA GAA TTC GCC CCG GCC TGG

TAC AC-3' and 5'-TAA GCT TGG CAC GGC TGT CCA AGG A-3') in GeneAmp PCR buffer (10 mM tris-hcl pH 8.3, 50 mM KC1, 1.5mM MgC12 and 0.001% gelatin, Perkin Elmer, Norwalk, CT), 10% DMSO, and AmpliTaq DNA polymerase (1.25U; Perkin Elmer, Norwalk, CT) with 2'- deoxynucleoside-3'-triphosphates (1.875mM; Pharmacia, Piscataway, NJ). The reaction yielded 244 base pair fragments. The PCR reaction had an initial melting temperature of 94°C; 30s), annealing (65°C; 30s), and extension (72°C; 30s). A final extension period of 7 min at 72°C followed the last cycle. PCR products were identified on a 2.2% agarose gel that was stained with ethidium bromide. After PCR amplification, 10 U of HhaI (New England Biolabs, Beverly MA) was added directly to each 40µl reaction for digestion at GCGC sites (3hr, 37°C). Digests were loaded onto a 10% (w/v) nondenaturating polyacrylamide gel (1.5mm thick x 25 cm long), run at 150 V for approximately 3 hr, stained with ethidium bromide and visualized by UV light. The assay was validated by confirming a polymorphic inheritance pattern in seven human family lines, encompassing three generations each (data not shown; NIGMS Human Genetic Mutant Cell Repository, Coriell Institute, Camden, NJ).

Triglycerides were analyzed by a manual colorimetric method [39], and total cholesterol and HDL cholesterol were assessed using a spectrophotometric kit method (Sigma Chemical, St. Louis, MO). LDL cholesterol was calculated by Friedewald's formula [40] : LDL_{CHOL} = $Total_{CHOL}$ - HDL_{CHOL} - (TRIG/5).

Statistical Analyses Descriptive analyses included Student t-tests of means for cases and controls for serum lipids, lifestyle, reproductive, and dietary variables, and chi square tests for categorical variables. Unconditional logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs). ORs were adjusted for potential confounders, including age, education, family history of breast cancer, parity, body mass index, age at first

pregnancy, menopausal status, and, where appropriate, serum cholesterol. Covariates were only included in the final regression model if they were established risk factors in these data or changed the observed risk estimate by at least 15 percent. In subgroup analyses for women with the apoE 2 genotype, we used a reduced logistic regression model, adjusting for age and menopausal status, based on the small number of women in this group.

We were concerned that serum lipid levels for breast cancer cases would be affected by post surgery treatment. However, blood samples of most cases were obtained before additional treatment; a small proportion provided the blood sample after chemotherapy (11%), novodex (9%), or radiation (2%) treatment. Serum lipid levels in these individuals were comparable to those observed among cases who had not received such treatment (data not shown). Furthermore, we initially included treatment into the logistic regression model, but inclusion did not affect the observed risk estimates, neither did exclusion of women who received adjuvant treatment from these analyses.

The effect of apoE genotype was examined by classifying participants as apoE 3 (homozygous for ε3 allele), apoE 2 (homozygous or heterozygous for ε2 allele), and apoE 4 (homozygous or heterozygous for ε4 allele). Eleven participants with the ε2, ε4 genotype were excluded from the logistic regression analyses. Exclusion was based on the counteracting biological effects of the ε2 and ε4 alleles in the context of our study hypotheses. Dietary fat and cholesterol intakes, as well as serum lipid concentrations were examined by dividing the sample into tertiles, based on the distribution of these variables in the control group. Logistic regression analyses were performed for premenopausal and postmenopausal women separately, but risk estimates did not differ substantially between these groups. All ORs reported here were based on a combined sample of pre- and postmenopausal women, adjusted for menopausal status.

Results

The distribution of apoE genotype in the total sample and by disease status is shown in Table 1. Regardless of disease status, nearly all participants (96 percent) carried at least one ε3 allele, 22 percent carried an ε4 allele, and 10 percent carried an ε2 allele, consistent with a recent report based on Caucasian women [41]. Table 1 displays mean serum lipoproteins by apoE genotype. In general, participants with the ε2, ε3 genotype tended to have lower serum triglycerides, total cholesterol and LDL cholesterol levels and higher HDL cholesterol levels, than participants homozygous for ε3, and participants with the ε4, ε3 alleles, respectively. This pattern was more apparent for the total sample and breast cancer cases and less apparent for controls.

Characteristics of breast cancer cases and community controls are shown in Table 2. The two groups were similar with respect to mean age, years of education, body mass index, age at menarche and menopause (postmenopausal women), and parity. Cases had a slightly older age at first birth, tended to lactate for shorter duration, and had a greater likelihood of having a family history of breast cancer and a personal history of benign breast disease. There were no differences between cases and controls with regard to mean levels of total cholesterol, HDL cholesterol, LDL cholesterol, or triglycerides, nor did the groups differ by mean intake of dietary fat (g/month) of dietary cholesterol (mg/month) (Table 3).

Risk of breast cancer in association with apoE genotype is shown in Table 4. Compared to women with the apoE 3 genotype (referent), there was no difference in risk for women with the apoE 2 genotype (adjusted OR=1.0; 95% CI 0.91-1.64) or women with the apoE 4 genotype (adjusted OR=0.97; 95% CI 0.63-1.54). These results were not substantially altered when the sample was divided into subgroups of women with lower (< median in the controls) or greater (> median in the controls) dietary in take of total fat or cholesterol (Table 4).

Table 5 displays the associations between serum lipoproteins and breast cancer risk in the total sample, as well as among subgroups of women defined by apoE genotype. Women with higher serum levels of total cholesterol and HDL cholesterol were not a greater risk of breast cancer compared to women with lower serum levels. These associations were not substantially different when apoE genotype was considered (Table 5). Elevated LDL cholesterol levels were associated with inverse associations in the total sample (adjusted OR=0.72; 95% CI 0.46-1.13), and more pronounced among women with the apoE 3 genotype (adjusted OR=0.56-; 0.33-0.97). No associations between LDL cholesterol and risk were observed for women with apoE 2 and apoE 4 (Table 5). In the total sample, there was some evidence that women with the highest levels of serum triglycerides had an increase in risk (adjusted OR=1.63; 95% CI 1.03-2.59) compared to those with the lowest levels. There was no strong evidence for such an association among women with apoE 3 or apoE 2, but among women with apoE 4, elevated triglycerides levels were strongly associated with risk (adjusted OR=4.69; 1.49-14.7).

Discussion

To our knowledge, this is the first study to examine associations between serum lipoproteins, apoE genotype, and breast cancer risk. Serum levels of total, HDL, and LDL cholesterol were not associated with risk in these data; stratification by apoE genotype did not substantially affect these observations. There was no direct effect of the apoE genotype on breast cancer risk, nor did risk estimates differ among women with lower or higher intake of dietary fat and cholesterol. However, we observed evidence for greater risk of breast cancer for women with elevated triglyceride levels, particularly among women with the apoE 4 genotype.

Previous studies have reported increased risk of breast cancer in association with elevated triglyceride levels [29-33, 35, 42, 43], and biologic mechanisms for this association have been

postulated. Increased triglyceride levels have been shown to be associated with decreased levels of sex hormone binding globulin, resulting in elevated levels of free estradiol, and subsequently increased risk of breast cancer [44]. Alternatively, hypertriglyceridemia could be related to breast cancer risk, due to its association with insulin resistance [45, 46]. Insulin resistance is also linked to increased sex steroid levels in association with decreased sex hormone binding globulin levels, as well as with increased bioactive levels of insulin-like growth factor I, which may act synergistically with estrogen in promoting breast cancer [47].

In this study we found elevated triglyceride levels to be associated with breast cancer risk among women with the apoE 4 genotype, which has been shown to be related to triglyceride levels in previous studies [48-50]. In these data, individuals with the apoE4 genotype had higher triglyceride levels than those with the apoE 3 genotype, and this effect was more pronounced among cases than controls (Table 1). Thus, the observed strong risk elevation among women with the apoE 4 genotype may be an effect of reduced triglyceride clearance from plasma, resulting in continuous elevated concentrations, which could result in decreased sex hormone binding globulin levels.

Several methodological issues need to be considered in interpreting these findings. As in most epidemiologic studies, we cannot rule out the potential effect of bias on our results. Non-participation among cases and controls may have biased these findings. For the cases, the primary reason for non-participation in the study was physicians refusing to allow us to contact their patients. It is possible that this lack of inclusion reflects physician rather than patient characteristics, but we could not verify whether or not this was true. Among the controls, we do have some evidence that at least for dietary intake, there were no differences among participants and those who did not participate [36, 51]. There was further lack of participation in the blood draw. Those providing blood were generally similar to those who did not. Further, non-

participation is probably is not related to apoE genotype. The allelic frequencies of apoE were similar to those observed in a large sample of Caucasian women [41]. Sample size was a limitation in this study, as in many molecular epidemiologic studies. Our findings were based on small numbers, resulting in possibly unstable risk estimates. It is possible that the association between apoE genotype, triglycerides, and breast cancer risk may be a result of sampling variation.

An inherent problem in case-control studies utilizing biological specimen relates to the possibility that the disease process or treatment may have affected the measurements in cancer cases. There was some speculation that blood lipid levels could be affected by surgery as part of the metabolic and neuroendocrine response to the procedure [52], but a recent study demonstrated that triglyceride levels were almost identical in blood samples obtained before and after breast cancer surgery [32]. There is evidence that blood lipid levels can be affected by chemotherapy and tamoxifen treatment [53-55]. However, these results were unlikely to be affected by alterations of serum lipoprotein levels as a function of cancer treatment, due to the following observations: a) the vast majority of cases have not undergone adjuvant treatment at the time of blood draw, b) lipoprotein levels of those who have received chemotherapy were similar to the levels of women without such treatment, and c) observed risk estimates based only those cases with pre-adjuvant therapy did not differ from those observed in the total sample (data not shown). However, the possibility remains that the observed differences in the blood triglyceride levels for the cases and controls were the result of the disease process and not an indicator of exposure. Furthermore, there is substantial intra-individual variability in serum triglyceride concentrations [56]. Although it is unlikely that this variability was related to disease status, it is possible that the triglyceride blood values from only one point in time did not reflect a stable measurement of triglyceride levels in this population.

In summary, in this case-control study women with elevated triglyceride levels were at greater risk of breast cancer than those with lower levels. This effect was more pronounced among women with the apoE 4 genotype. Overall, apoE genotype was not related to risk of breast cancer in crude analyses or when analyses were stratified by fat and cholesterol intake. These results indicate that metabolism of dietary lipids by apoE could modify the association between consumption of dietary fat, particularly sources of triglycerides, and breast cancer risk.

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Quantitation of carcinogen-DNA adducts by ^{14}C-postlabeling methods

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RUNNING TITLE

^{14}C-postlabeling of DNA adducts

KEY WORDS

molecular epidemiology, carcinogenesis, ^{14}C-postlabeling, DNA adduct, benzo[*a*]pyrene

ABBREVIATIONS

AMS, accelerator mass spectrometry; AP, alkaline phosphatase; BPDE, benzo[*a*]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide; BPdG (Rttr), 7R,8S,9R-trihydroxy-10S-(N^2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene; CD, circular dichroism; dG, deoxyguanosine; DMSO, dimethylsulfoxide; LC-MS, liquid chromatography-mass spectrometry; LSC, liquid scintillation counting; MeIm, 1-methylimidazole; NP1, nuclease P1; PB, phosphate buffer; RP-HPLC, reverse phase-high performance liquid chromatography; SVPD, snake venom phosphodiesterase; TEA, triethylamine; TFE, trifluoroethanol; THF, tetrahydrofuran; TLC, thin layer chromatography

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ABSTRACT

Quantitation of carcinogen-DNA adducts provides an estimate of the biologically effective dose of a chemical carcinogen reaching the target tissue. In order to improve exposure-assessment and cancer risk estimates, we are developing an ultrasensitive procedure for the detection of carcinogen-DNA adducts. The method is based upon postlabeling of carcinogen-DNA adducts by acetylation with ^{14}C-acetic anhydride combined with quantitation of ^{14}C by accelerator mass spectrometry (AMS).

For this purpose, adducts of benzo[*a*]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (BPDE) with DNA and deoxyguanosine (dG) were synthesized. The most promutagenic adduct of BPDE, 7R,8S,9R-trihydroxy-10S-(N^2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPdG), was HPLC purified and structurally characterized. Postlabeling of the BPdG adduct with acetic anhydride yielded a major product with a greater than 60% yield. The postlabeled adduct was identified by liquid chromatography-mass spectrometry as *pentakis*(acetyl) BPdG (AcBPdG). Postlabeling of the BPdG adduct with ^{14}C-acetic anhydride yielded a major product coeluting with an AcBPdG standard. Quantitation of the ^{14}C-postlabeled adduct by AMS promises to allow detection of attomolar amounts of adducts. The method is now being optimized and validated for use in human samples.

INTRODUCTION

Epidemiological studies indicate that a substantial portion of human cancer derives from exposures to chemical carcinogens [1]. Most carcinogens form DNA adducts as a common pathway to mutagenesis [2,3]. The detection of carcinogen adducts in human tissues is a central strategy in molecular epidemiology [4] allowing: 1) estimates of the effective dose of a carcinogen in the target tissue; and 2) correlation of exposure with the incidence of disease.

Tracing the links from carcinogen exposure, DNA binding, resultant mutations and cancer contributes to an understanding of cancer etiology and the design of prevention strategies [5,6]. This task, however, is inherently difficult as humans are typically exposed to only low level complex carcinogen mixtures [7]. Difficulties for carcinogen-DNA adduct detection arise, in part, due to insufficient assay sensitivity, specificity, and reproducibility [8,9]. Detection methods based on immunohistochemistry or ^{32}P-postlabeling may be sensitive enough [10], but lack the desired specificity unless combined with micropreparative steps [11]. These additional analytical steps (e.g., multidimensional TLC) tend to reduce sensitivity and increase labor and/or cost beyond acceptable limits [12,13].

It is expected that molecular epidemiological studies based on improved detection and quantitation of DNA-adducts (and other biomarkers) will provide new, invaluable information for early detection and prevention of cancer. This report describes the development of an alternative method for the detection of benzo[*a*]pyrene-DNA adducts based on postlabeling with ^{14}C-acetic anhydride and quantitation of the ^{14}C by accelerator mass spectrometry (AMS).

MATERIALS

(±)-BPDE, (+)-BPDE, and (–)-BPDE were purchased from Chemsyn (Lenexa, KS). (Milwaukee, WI). AP, DMSO, SVPD, and dG were from Sigma (St. Louis, MO). THF/MeIm was from Perkin-Elmer/ABI (Foster City, CA). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). [$^{14}C_4$]acetic anhydride was from DuPont-NEN (Billerica, MA). Pyridine was distilled over sodium and stored at 24 °C in sealed glass ampules. DMSO was distilled under reduced pressure.

METHODS

Synthesis of BPdG (Rttr)

Two methods were used to obtain sufficient quantities of BPdG standards. Method 2., based on derivatiozation of dG with BPDE, has higher yields than the method based on derivatization of DNA.

Method 1. Calf thymus DNA (3 mg) was reacted with (+)-BPDE (0.5 mg) or racemic (±)-BPDE (0.5 mg) in 3 ml of 0.1M PB, *p*H 7.5, containing 0.1 ml DMSO and 0.3 ml C_2H_5OH at 37 °C for 12 h. The reaction was extracted with diethyl ether, ethyl acetate, and *n*-butanol (3 x 10 ml each). The DNA was precipitated from the aqueous layer with 0.3 M sodium acetate, *p*H 5.2, containing 2.5 volumes of C_2H_5OH. The precipitate was washed with 7:3 C_2H_5OH-H_2O, dissolved in 0.1 M sodium acetate, *p*H 5.3, with 0.2 mM $ZnCl_2$, and digested with nuclease P1 (77 U/mg) for 3 h at 37 °C. The *p*H of the buffer was adjusted with 0.3 M Na_2CO_3 to *p*H 8.5, and made 2 mM in $MgCl_2$. Alkaline phosphatase, 17 U/mg, and SVPD, 0.9 U/mg, were added and the reaction was further incubated for 3 h at 37 °C. The digest was

cleaned on a C_{18} SepPak with H_2O (30 ml) and eluted with 3 ml of CH_3OH. The methanolic eluent was concentrated on a Speedvac and reaction products were separated by HPLC using method A (see below).

Method 2. (+)-BPDE or racemic (±)-BPDE (0.5 mg) was dissolved in 100 µl of DMSO and reacted with dG (70 mg) in 5 ml of TFE containing 63 µl of TEA and heated to 37 °C for 5 h. The solvent was evaporated on Speedvac. The resulting mixture was resuspended in distilled H_2O and extracted with *n*-butanol (4 x 10 ml). The pooled organic layers were pooled, washed with H_2O (3 x 50 ml), and concentrated on a rotary evaporator. The residue was dissolved in CH_3OH and separated by HPLC using method A (see below).

Acetylation of BPdG with acetic anhydride

The yields of acetylation was compared in several solvent systems. The reaction has high yields in both pyridine and THF/MeIm, but the second method is faster and more efficient with low concentrations of starting material.

Method A. BPdG (Rttr) (10 nmol) was acetylated for 2 h at 35 °C with cold acetic anhydride (3 µl) in 20 µl of anhydrous pyridine. The reaction was terminated by the addition of 20 µl of 50% aqueous CH_3OH and evaporation of the solvents to dryness on a Speedvac. The product of the reaction was purified by HPLC method C (see below). The separated peaks were collected and analyzed by LC-MS.

Method B. Acetic anhydride (3 µl) was dissolved in 400 µl of THF/MeIm. An aliquot of 20 µl was added to HPLC-purified BPdG (1-100 pmol). The reaction was carried out for 20 min

at 22 °C, stopped with 20 µl of 50% aqueous CH_3OH, and evaporated to dryness on a Speedvac. The reaction products were analyzed by HPLC method C and the major product was further analyzed by LC-MS.

Acetylation of BPdG with [$^{14}C_4$]acetic anhydride

Method A. BPdG (Rttr) (2 nmol) was acetylated for 2 h at 35 °C with 8.4 mCi/mmol [$^{14}C_4$]acetic anhydride (3µl) in 12 µl of anhydrous pyridine. The reaction was terminated by evaporation of the solvent on a Speedvac. The concentrated reaction mixture was dissolved in 200 µl of CH_3OH and evaporated to dryness three times. The product of the reaction was purified by HPLC method C (see below). The peak corresponding to *pentakis*(acetyl) BPdG was collected and the column was washed with 200 ml of THF, 200 ml of H_2O, and 200 ml of CH_3OH. Aliquots of the purified [^{14}C]AcBPdG were reinjected on the washed HPLC column. The fractions co-eluting with AcBPdG standard were analyzed by AMS for ^{14}C-content (see below).

Method B. [$^{14}C_4$]acetic anhydride (3µl) was dissolved in 400 µl of THF/MeIm. A 20 µl aliquot was added to the HPLC-purified Rttr stereoisomer of BPdG (10 pmol) under a controlled anhydrous atmosphere in a polycarbonate reaction chamber (Coy Laboratories, Grass Lake, MI). The reaction was carried out for 20 min at 22 °C, stopped by addition of 50% aqueous CH_3OH (20 µl), and evaporated to dryness on a Speedvac. The reaction products were loaded on a Waters C18 SepPak cartridge (100 mg) together with 5 pmol of cold AcBPdG standard. The SepPak was washed with 0.03% aqueous CH_3CO_2H (50 ml), 10% aqueous CH_3OH (50 ml), 30% aqueous CH_3OH (50 ml), and 20 mM ammonium acetate,

*p*H 7.4 (50 ml). [^{14}C]AcBPdG was eluted with CH_3OH (2 ml). Aliquots of the methanolic eluent were analyzed by HPLC, method C, and the ^{14}C content of the HPLC fractions was measured by AMS (see below).

Accelerator mass spectrometry

Measurement of the radiocarbon content of the samples was carried out using accelerator mass spectrometry (AMS) [14,15]. AMS measures the amount of radioisotope, e.g., ^{14}C relative to a stable isotope of the same element. In this study, ^{14}C was measured relative to ^{13}C and then normalized to the ratio of $^{14}C/^{12}C$ using the Australian National University sugar standard [16] as reference. For AMS analysis, HPLC samples were converted to graphite by a two step process involving combustion of the samples to CO_2 followed by reduction to filamentous graphite, as described previously [17]. Because the complete process to graphite works best with 1-2 mg of total carbon, and to insure a well-known and constant amount of carbon in each HPLC fraction, 2 mg of tributyrin (providing *ca.* 1 mg of carbon), well characterized with respect to radiocarbon content, was added to fractions as a carrier carbon prior to combustion. The samples were measured as graphite and converted to amol radiocarbon/mg sample by subtracting carbon-14 contribution from control material and carrier carbon [14,17].

Electrospray mass spectrometry

Mass spectrometric determinations were carried out on Perkin-Elmer/Sciex API 1 spectrometer equipped with an atmospheric pressure ionization source and an IonSpray interface which was maintained at 5 kV. The orifice was maintained at 70 V, high purity N_2

flowing at 0.6 L/min served as curtain gas, and high-purity air maintained at 40 psi was used for nebulization. Samples were introduced using a Hewlett-Packard 1090 Series II liquid chromatograph equipped with a diode array 1040A detector.

The analytes (BPdG or AcBPdG) were introduced into the mass spectrometer without column separation and without splitting of the effluent. As little as 2 pmol of analyte was injected into the ionization source in a 40 µl/min flowing stream of 50% aqueous CH_3CN containing 0.05% CF_3CO_2H. Mass spectra were acquired every 6-12 s over the range of *m/z* 300 to 1000 (m/z 0.1 resolution). Analytes were detected as their $[M+H]^+$, $[M+K]^+$, or $[M+Na]^+$ ions.

HPLC separation of adducts

Analytes were injected with a Waters 717Plus autosampler into a Hewlett-Packard 1050 Series liquid chromatograph equipped with a diode array 1040A detector.

Method A. The separation of BPdG (Rttr) was achieved with a C_{18} column (Beckman Ultrasphere ODS, 5 µm particle size, 10 x 150 mm) using a gradient of CH_3OH (solvent A)-H_2O (solvent B) (0-3 min, 40% A; 3-5 min, linear gradient to 50% A; 5-22 min, linear gradient to 55% A, 22-26 min, linear gradient to 99% A; 26-30 min isocratic at 99% A) as the mobile phase at a flow rate of 3 ml/min with UV monitoring at 279 and 344 nm. Under these conditions the retention time of BPdG (Rttr) was 18 min.

Method B. Acetylated BPdG was separated from other analytes on a C_{18} column (Beckman Ultrasphere ODS, 5 µm particle size, 4.6 x 250 mm) using a gradient of CH_3OH (solvent A)-

H_2O (solvent B) (0-20min, 70% A; 20-22 min, linear gradient to 90% A; 22-29min, isocratic at 90% A; 29-30 min, linear gradient to 100% A; 30-35 min, isocratic at 100% A) as the mobile phase at a flow rate of 1 ml/min with UV monitoring at 279 and 344 nm. Under these conditions the retention time of *pentakis*(acetyl) BPdG (Rttr) was 22.8 min.

Method C. A longer gradient of CH_3OH (solvent A)-H_2O (solvent B) (0-10min, 20% A; 10-13 min, linear gradient to 50% A; 13-25min isocratic at 50% A; 25-28 min, linear gradient to 70% A; 28-38 min, isocratic at 70% A; 38-40 min, linear gradient to 90% A; 40-50 min, isocratic at 90% A; 50-53 min, linear gradient to 100% A; 53-60 min, isocratic at 100% A) was used for cleanup of ^{14}C-acetylated BPdG. The retention time of *pentakis*(acetyl) BPdG (Rttr) was 44.5 min under these conditions.

UV-Vis and CD spectra

All spectrophotometric measurements were carried out using a Beckman DU640 spectrophotometer. The concentration of BPdG was measured at 279 nm using ε = 40,984 M^{-1} cm^{-1}. CD spectra were recorded on a JASCO J-500A spectropolarimeter.

RESULTS

1. **Synthesis and identification of BPdG (Rttr)**

Derivatization of DNA with (+)-BPDE or (±)-BPDE yielded one major product as previously described [18]. The product was purified from the enzymatic DNA digest by HPLC and characterized. The mass spectrum of the purified adduct was consistent with the structure of a BPDE adduct with dG (BPdG) (Figure 1). CD spectropolarimetry showed that the product

was the Rttr stereoisomer of BPdG [18] (Figure 1). Synthesis of BPdG from dG and (±)-BPDE was carried out in TFE/TEA [19]. The reaction afflorded a product with the same spectral characteristics as the BPdG adduct isolated from BPDE-derivatized DNA (data not shown), but in higher yield. This method was used for the preparation of standards of the BPdG adducts. The adducts were used for the optimization of postlabeling reactions and as chromatographic standards.

2. **Acetylation of BPdG (rttr)with cold acetic anhydride**

Postlabeling of the BPdG (Rttr) adduct by acetylation with acetic anhydride was initially carried out as described previously [18]. Acetylation in pyridine yielded one major product in > 60 % yield. The product was isolated by HPLC. Its protonated quasimolecular ion (*m/z* 779; electrospray MS) of the major product was consistent with addition of five acetyl groups to BPdG (Rttr), i.e., *pentakis*(acetyl) BPdG (AcBPdG) (Figure 2). The AcBPdG was further used as a standard for HPLC separations of ^{14}C-AcBPdG.

Use of THF/MeIm as a solvent/base pair gave higher postlabeling yields when picomolar amounts of the BPdG adduct were acetylated [20]. *pentakis*(Acetyl) BPdG was also the major product under these conditions as verified by LC-MS, but the reaction was faster and more efficient (data not shown).

3. **Acetylation of BPdG (Rttr) with [$^{14}C_4$]acetic anhydride**

Acetylation of BPdG (Rttr) at the nanomolar scale with excess [$^{14}C_4$]acetic anhydride yielded a major peak in the (accelerator mass spectrometric) AMS profile of the HPLC fractions

(Figure 3). The AMS profile of the HPLC fractions was prepared by analyses according to established procedures [14,17]. The radiolabeled product co-eluted with the cold AcBPdG standard. Aliquots of the isolated [^{14}C]AcBPdG product were further analyzed by HPLC after extensive washing of the column to remove background ^{14}C (i.e., carryover of ^{14}C from the labeling reaction). Chromatographic analyses of the aliquots with AMS quantitation of [^{14}C]AcBPdG allowed construction of a theoretical dose-response curve for detection of the postlabeled adduct (Figure 3). The detection limit under these conditions was 1 fmol of adduct, even though the fractions showed a 10-fold higher level of background ^{14}C than expected (Figure 3).

An additional cleanup step with a C_{18} SepPak cartridge preceding the HPLC separation eliminated the increased background ^{14}C. Postlabeling of picomolar amounts of BPdG (method 2) combined with SepPak cleanup and HPLC separation yielded AMS profiles with the major peak co-eluting with AcBPdG standard (Figure 4). Analyses of aliquots of this reaction allowed construction of a new dose-response curve for detection of the postlabeled [^{14}C]AcBPdG (Figure 4). The detection reached the theoretical limit of 100 attomoles of adduct using these conditions.

DISCUSSION

Cancer results from an as yet unidentified interplay of exogenous and endogenous exposures and genetic susceptibilities [4,5]. Many ongoing studies in our laboratory [21,22] and elsewhere [23,24] examine the contribution of genetic polymorphisms to the variability of function in both 'caretaker' (metabolism, detoxification, DNA repair) and 'gatekeeper' (cell

cycle control, apoptosis) cancer susceptibilty genes [25]. Carcinogen-DNA adducts serve as biomarkers that link genetic susceptibility with an exposure [8]. For example, in lung [26], bladder [27,28], and other tissues [29], DNA adduct levels are higher in persons with hypothesized 'at risk' genetic variants. This shows that the carcinogen-DNA adducts estimate the total burden of a particular exposure in the target tissue and suggest a link to cancer risk [26,30].

In our previous studies, chemical-specific measurements of carcinogen-DNA adducts in human tissue reached the detectable limit in approximately 25% of examined samples [12]. This prompted us to develop a more sensitive method with retained chemical specificity for high throughput epidemiological studies. The chosen approach combines postlabeling of carcinogen-DNA adducts with [^{14}C]acetic anhydride with quantitation of ^{14}C by AMS (Scheme 1). AMS is an ultrasensitive ^{14}C detection method with documented sensitivity of zeptomole (10^{-20} mole) quantities of ^{14}C [14]. This leads to a theoretical detection limit of 1 adduct in 10^{12} to 10^{14} nucleotides based on studies with ^{14}C-labeled carcinogens [31]. This limit therefore offers a 1000-fold improved sensitivity over currently available methods [9]. We are attempting to combine this unmatched sensitivity of AMS with the versatility of the postlabeling methods. This novel approach should be easily adaptable to a wide range of compounds and expand the scope of biological applications of AMS. The detection of benzo[*a*]pyrene adducts is particularly promising because the postlabeling by acetylation with [^{14}C]acetic anhydride has an established chemical precedent [18].

The method is being developed on the most carcinogenic stereoisomer of the adduct formed by benzo[*a*]pyrene diolepoxide with deoxyguanosine which is the 7R,8S,9R-trihydroxy-10S-(N^2deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene, BPdG (Rttr). Postlabeling of this widely studied promutagenic adduct will allow comparison with established methods. The acetylation reaction yielded one major product, *pentakis*(acetyl) BPdG (AcBPdG), in high yield. Postlabeling by acetylation with [^{14}C]acetic anhydride was analogous to the labeling with cold acetic anhydride as expected. The major peak of ^{14}C detected by AMS co-eluted with the cold AcBPdG standard in the HPLC profile (Figures 3a and 4a). The area under the peak was proportional to the amount of postlabeled adduct injected on the column (Figure 4b). It is important to separate the excess ^{14}C from the ^{14}C-postlabeled adduct prior to the HPLC analyses (Figure 3b). Impractical amounts of solvent (> 500 ml) had to be used for column washing when > 100 dpm of ^{14}C was applied to the HPLC column. The cleanup was still incomplete after a wash with 600 ml of solvents when > 10^6 dpm of ^{14}C was loaded on the column (Figure 3b). The excess ^{14}C-label was therefore washed out on a C_{18} SepPak cartridge prior to the HPLC analysis. The procedure provided sufficient cleanup without affecting recovery (Figure 4). The recovery tested with synthesized ^{3}H-AcBPdG was always greater than 90% (data not shown). The current sensitivity in the attomolar range promises excellent sensitivity for the detection of adducts in human DNA, provided that the reactions/cleanup proceed without additional complications at smaller scale. Optimization of the washing step and of the reaction at femtomolar and attomolar starting amounts of adduct are underway.

This report summarizes the development of a novel postlabeling method based on acetylation with ^{14}C combined with AMS quantitation. The presented preliminary results are encouraging.

Two micropreparative steps, immunoaffinity chromatography for isolation of the adduct of interest from DNA and HPLC for separation of postlabeled adducts, assure chemically specificity. The assay also promises excellent sensitivity.

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FIGURE LEGENDS

Figure 1. Characterization of the major BPdG stereoisomer.

A. CD spectra of the HPLC-purified BPdG (Rttr) are identical with previously published CD spectra

B. The quasimolecular ion determined by electrospray mass spectrometry is consistent with that of the BPdG adduct.

Figure 2. Characterization of the BPdG postlabeled by acetylation (AcBPdG).

A. The HPLC profile (monitored at 344 nm) shows one major product with a retention time of 22.9 min.

B. The mass spectrum of the HPLC purified peak (22.9 min) is consistent with the *pentakis*(acetyl) BPdG adduct.

Figure 3. Analysis of the ^{14}C-acetylated BPdG (nanomolar scale).

A. HPLC profile of the [^{14}C]AcBPdG with AMS detection. The major ^{14}C-peak, RT 44.2 min, coelutes with the synthesized AcBPdG standard. Modern is defined as 5.9×10^{10} $^{14}C/g$ of carbon (approximately the concentration of ^{14}C in the atmosphere in 1950).

B. Dose-response of [^{14}C]AcBPdG aliquots of the major peak. The graph (log/log scale) shows that the background in the samples is about ten-fold higher than the background ^{14}C in the tributyrin solvent.

Figure 4. Analysis of the ^{14}C-acetylated BPdG (picomolar scale).

A. HPLC profile of the [^{14}C]AcBPdG with AMS detection after preparatory cleanup on a C18 seppak cartridge. The major ^{14}C-peak (44.2 min) coelutes with the synthesized AcBPdG standard.

B. Dose-response of ^{14}C-AcBPdG aliquots of the major peak. The background in the samples is similar to the background ^{14}C in the tributyrin solvent, i.e., about 0.1 Modern. The projected sensitivity is about 100 amol of adduct.

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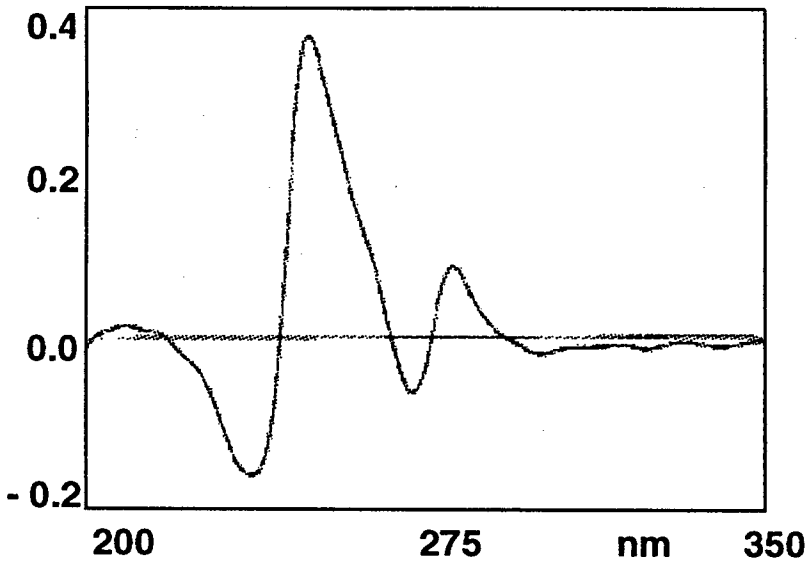
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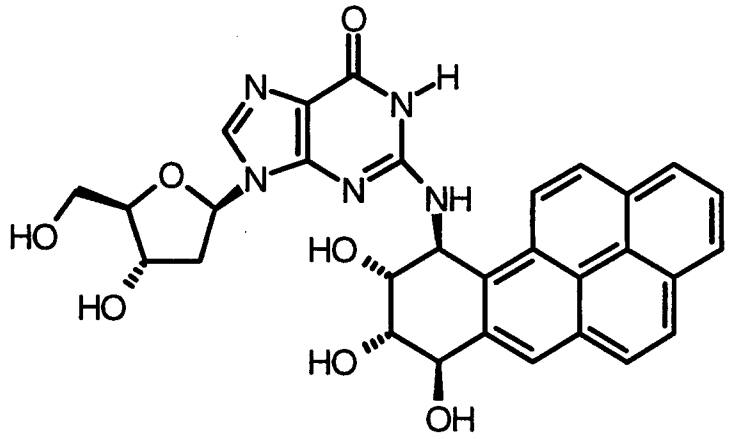
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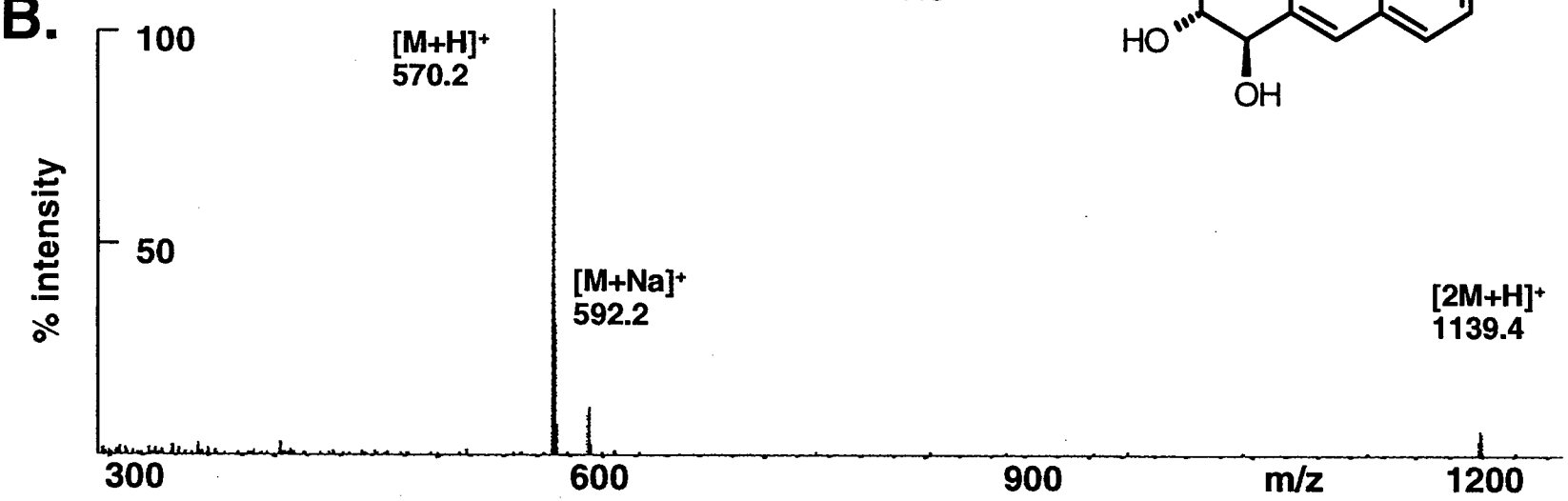
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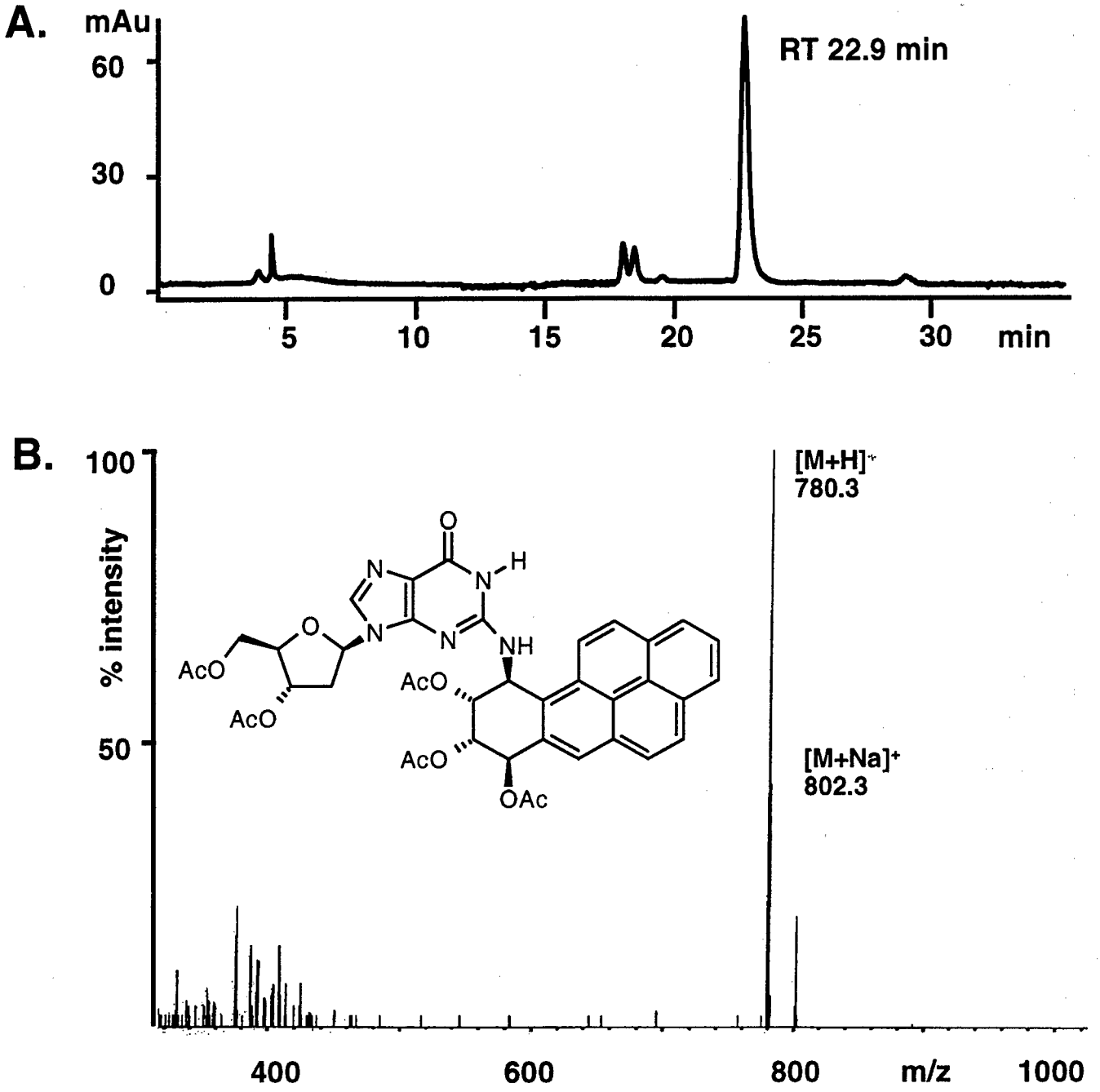
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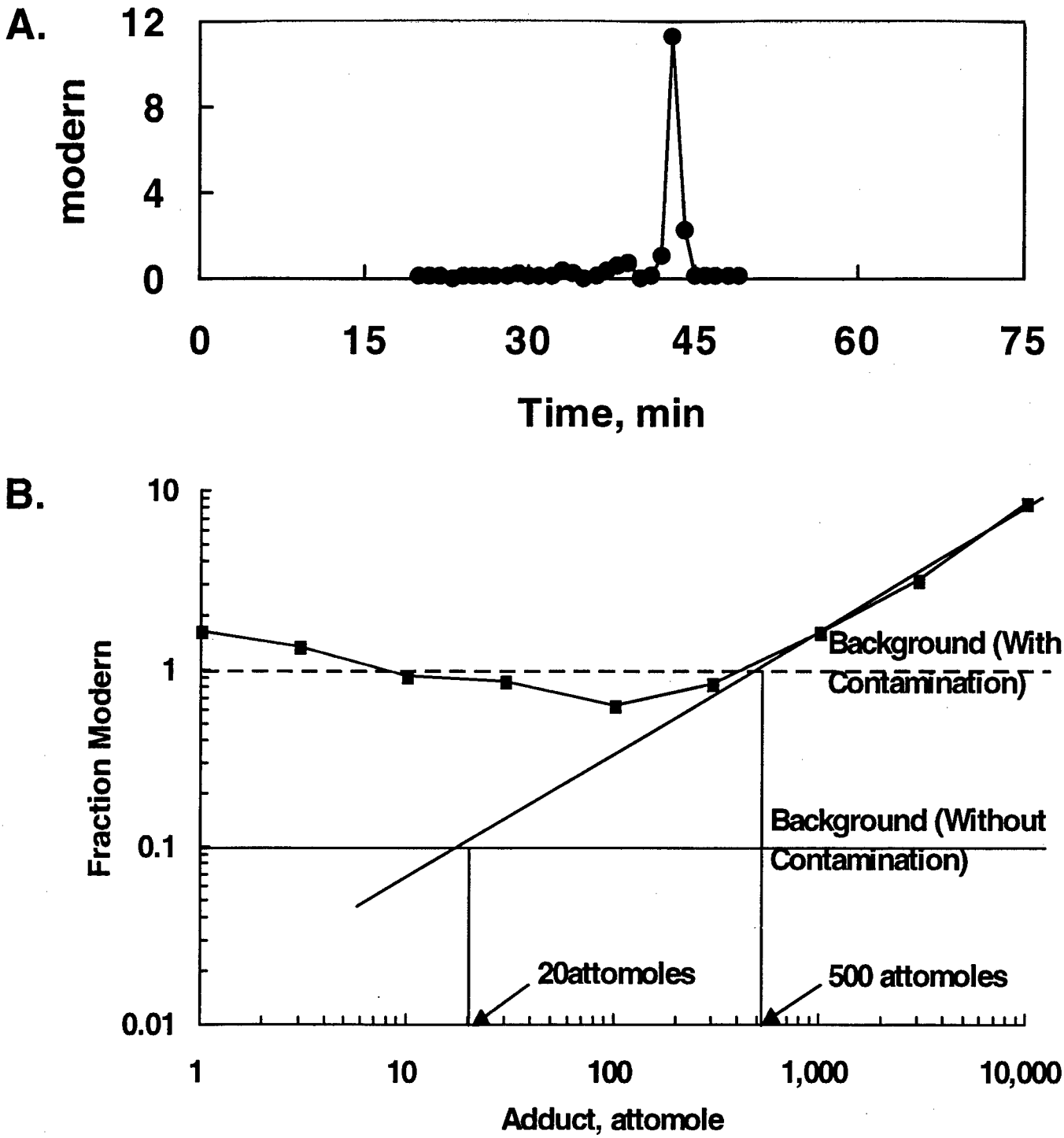
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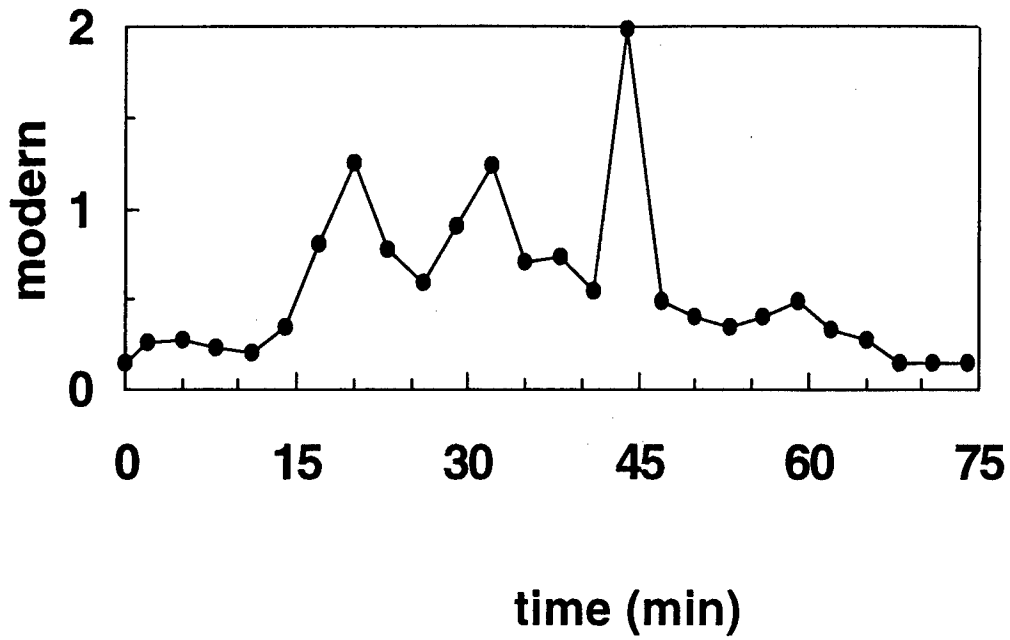
500 attomoles
1
10
100
1,000
10,000
Adduct, attomole

A.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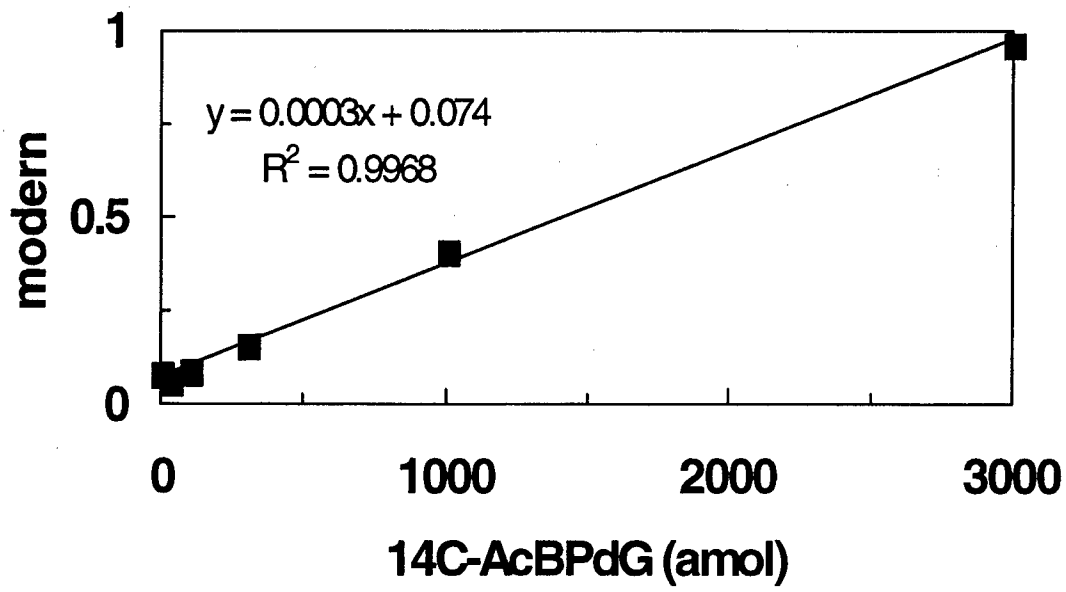
1
0
modern
0
15
30
45
60
75
time (min)

B.


1
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0
modern
y = 0.0003x + 0.074
R2 = 0.9968
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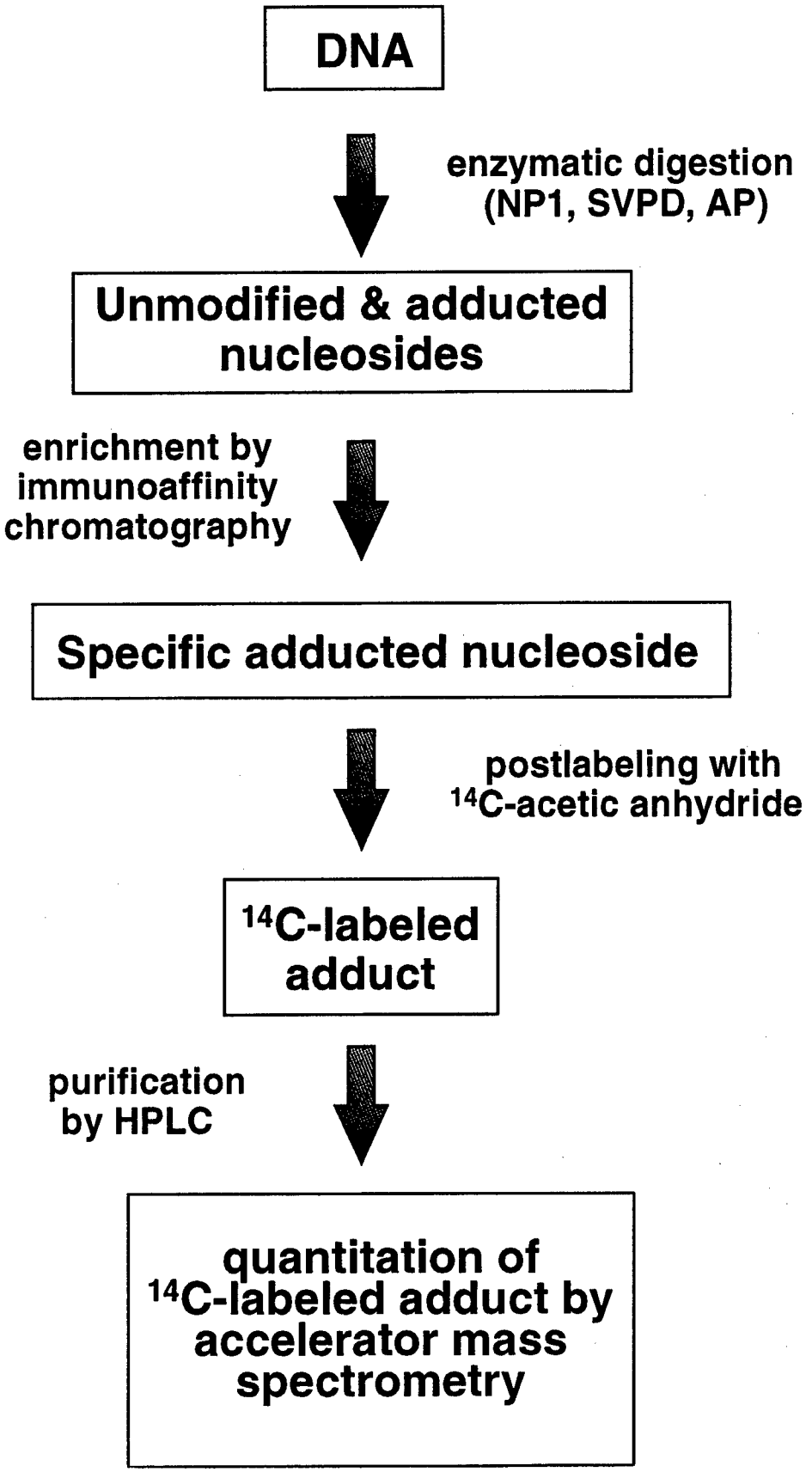
1000
2000
3000
14C-AcBPdG (amol)

Proposed scheme of ^{14}C-postlabeling



APPENDIX P – Association of dopamine transporter (DAT1) and DRD2 receptor genes with cigarette smoking

Lerman, C., Caporaso, N. E., Main, D., Audrain, J., Bowman, E. D., Lockshin, B., Boyd, N. R., Shields, P. G.: Association of dopamine transporter (DAT1) and DRD2 receptor genes with cigarette smoking. Health Psychology., 18:14-20, 1999

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Association of Dopamine Transporter (*SLC6A3*) and *DRD2* Receptor Genes with Risk of Cigarette Smoking

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RUNNING HEAD: Genes and Smoking

Abstract

In a case-control study of 230 smokers and 230 race-matched nonsmokers, we investigated the risk of smoking associated with two genes affecting regulation of the neurotransmitter dopamine: the dopamine transporter gene (*SLC6A3*) and the D_2 dopamine receptor gene (*DRD2*). A gene-gene interaction was found wherein subjects with *DRD2* A2 genotypes and *SLC6A3* 9 genotypes were less than one-half as likely to be smokers (OR=0.44, 95% C.I.=0.27, 0.73, p=0.001). Moreover, smokers with *SLC6A3* 9 genotypes reported having been able to quit for a significantly longer period than all other smokers (480 $\pm$ 114 days versus 233 $\pm$ 48 days, respectively, p=0.05). This study provides the first evidence that *SLC6A3* may decrease the risk of cigarette smoking by more than one-half, and suggests that smoking may be influenced by an interplay among multiple genes affecting dopaminergic reuptake and receptor binding. A better understanding of genetic and neuropharmacologic factors contributing to smoking behavior will lead to improved strategies for smoking prevention and treatment.

Key words: smoking, genetics, dopamine

Introduction

Cigarette smoking is a major risk factor for cancer, atherosclerotic vascular disease, respiratory illness, and early mortality. Broad-based smoking cessation treatments have met with limited success, in part, because they have not accounted for individual differences in susceptibility to nicotine dependence (1). There is growing empirical support for the efficacy of pharmacologic smoking cessation treatments in conjunction with behavioral counseling (2). However, even with these more intensive approaches, only a subset of smokers are able to remain abstinent.

Propensity to smoking and difficulty refraining from smoking may be attributable, in part, to genetic factors (3,4). Several converging lines of evidence point to the neurotransmitter dopamine as a possible explanation for these genetic effects (5). As with other psychostimulants, the reinforcing properties of nicotine have been attributed to its effects on dopamine transmission (6,7). Nicotine has been shown to stimulate dopamine release (6,8) and to inhibit reuptake (9), thereby increasing levels of synaptic dopamine and satisfying the reward mechanism (7).

Two genetic polymorphisms might be important in dopamine regulation and nicotine dependence: 1) the dopamine transporter gene (*SLC6A3*), which governs the reuptake of dopamine from the neuronal synapse, and 2) the D_2 dopamine receptor gene (*DRD2*), which is a post-synaptic receptor. The *DRD2* A1 genotype has been associated with a reduced density of receptors (10), as well as with cigarette smoking (11,12), substance abuse (13), and alcoholism (14,15). However, reports of the alcoholism associations have not been consistent (16,17). The *SLC6A3* gene has not yet been examined for associations with smoking, and there are no studies that examined the interacting effects of these two genotypes.

Patients and Methods

Sample Selection and Composition. Subjects included 230 smokers, ages 18 and older, who reported smoking at least 5 cigarettes/day for at least one year. They were recruited for a free smoking cessation program through varied newspaper advertisements and flyers in Washington, DC and Philadelphia, PA. Two hundred thirty race-matched nonsmokers, who reported having smoked less than 100 cigarettes in their lifetimes, were recruited through similar mechanisms. Exclusion criteria were: under age 18; a personal history of cancer; undergoing treatment for drug or alcohol addiction, or presence of a psychiatric disorder which precluded informed consent.

Procedures. During a visit to the clinic, subjects completed an informed consent form and questionnaires assessing demographics and smoking history. All subjects donated blood for genetic analysis. Genotyping for *SLC6A3* was performed as described previously (18). The reaction conditions included an initial melting step (94°C; 4 min), followed by 35 cycles of melting (94°C; 1 min), annealing (65°C; 1 min) and extending (72°C; 1 min). The VNTR repeat was determined using a 4% agarose gel electrophoresis (3:1 Nusieve:Agarose). For the *DRD2* A1 genetic polymorphism, DNA was genotyped as described previously (19). The reaction conditions included an initial melting step (94°C; 5 min), followed by 35 cycles of melting (94°C; 1 min), annealing (58°C; 1 min) and extending (72°C; 1 min). A final extension step was used (72°C; 5 min). An aliquot (25 µl) was then subjected to *Taq*1 RFLP digestion according to the manufacturers instructions.

Statistical Analysis. As in previous studies, *DRD2* genotype was classified as the

presence or absence of the A1 allele (12-14) (i.e., A1/A1 +A1/A2 vs. A2/A2) and the *SLC6A3* genotype was classified as the presence or absence of the 9 allele (20) (i.e., 9/9 + 9/* vs. */*, where * connotes all other alleles). Associations between genotype and smoking, and possible interacting effects of *SLC6A3* and *DRD2,* were initially examined using Chi Square Tests of Association. Associations of genotype with continuous smoking variables (age at smoking initiation, prior quitting history, smoking rate) were examined using student T-tests. The main and interacting effects of the *SLC6A3* and *DRD2* genotypes were then examined in logistic and linear regression analyzes controlling for potential confounder variables (race, gender, age, BMI, alcohol intake [number of drinks of beer, wine, or hard liquor per week], and current use of psychotropic medication [antipsychotics, antidepressants, anxiolytics]). Odds ratios (ORs) and their 95% confidence intervals were calculated by unconditional logistic regression using SAS (Cary, N.C.). Controlling variables having $p \leq 0.20$ associations with smoking were retained in the final models.

Results

Descriptive Statistics. The sample included 230 smokers and 230 race-matched nonsmokers, 40% of whom were male and 60% were female. The racial composition was 89% Caucasian (n=408), and 11% African American (n=52). The average age was 33 years (range = 18-80 years). Among smokers, the average smoking rate was 22 $\pm$ 10 cigarettes/day.

The allelic and genotype frequencies for *SLC6A3* were similar to those reported in the literature for Caucasians and African Americans (20,21). However, a low frequency allele with 6 repeats (320bp) that has not been previously reported was found (EMBL #4101 41). The allelic frequencies for *SLC6A3* were in Hardy-Weinberg equilibrium, except for the group of African American smokers (χ^2 =4.66, p<.05). The allelic and genotype frequencies for the *DRD2* gene were also similar to previous reports (13, 15-17). These allelic frequencies were in Hardy-Weinberg equilibrium in both Caucasians and African Americans.

Univariate Associations of Genotypes with Smoking. As shown in Table 1, significantly fewer smokers than nonsmokers had *SLC6A3* 9 genotypes (46% vs. 56%; $OR_{smoking}$= 0.66, 95% C.I.=0.46, 0.97, p=0.03). The difference in the prevalence of the *SLC6A3* 9 genotype was evident in the Caucasian subjects (n=204; 48% vs. 58%; OR=0.65, 95% C.I.=0.44, 0.96, p=0.03), but not in African Americans (n=26; p=0.77). Overall, the *SLC6A3* 9 genotype was significantly less common in African Americans than in Caucasians (33% vs. 53%; χ^2 =7.93, p=0.005). There were no significant associations of *DRD2* genotype with smoking in either Caucasians or African Americans; although there was a slightly higher prevalence of *DRD2* A1 genotypes in smokers than in nonsmokers (37% vs. 32%). Overall, the *DRD2* A1 genotype was significantly

more common in African Americans than in Caucasians (54% vs. 35%; χ^2 =7.38, p=0.007).

As shown in Figure 1, the association of *SLC6A3* with smoking was modified by *DRD2* genotype. Among subjects not carrying the *DRD2* A1 allele (i.e., homozygous for *DRD2* A2), 41% of smokers had the *SLC6A3* 9 genotype compared with 61% of nonsmokers. In univariate analysis, this effect was significant in the overall study group ($OR_{smoking}$=0.45, 95% C.I.=0.28, 0.72, p=0.001) and also in the subset of Caucasian subjects ($OR_{smoking}$=0.46, 95% C.I.=0.28, 0.76, p=0.002). Among African American subjects, a similar effect was observed; 18% of smokers had the *SLC6A3* 9 genotype compared with 46% of nonsmokers. While these data indicate a stronger effect in this subset, statistical significance was not achieved, possibly due to the small sample size ($OR_{smoking}$=0.26; 95% C.I.=0.04, 1.7, p=0.15).

Multivariate Analysis of Genotypes with Smoking. The *DRD2* by *SLC6A3* interaction was tested in a logistic regression model controlling for body mass index, alcohol intake, and psychotropic medication use. Gender and age were also examined as covariates, but not retained in the final model because of lack of significant effects. The *DRD2* by *SLC6A3* interaction term was statistically significant (p=0.01). This interaction showed that, among subjects not carrying the *DRD2* A1 allele, those with the *SLC6A3* 9 genotype were less than half as likely as those without the 9 genotype to be smokers ($OR_{smoking}$=0.44, 95% C.I.=0.27, 0.73, p=0.001). In contrast, reduction in smoking risk associated with the *SLC6A3* 9 genotype was not observed in subjects who carried an *DRD2* A1 allele ($OR_{smoking}$ =1.23, 95% C.I.=0.64, 2.35, p=0.21). The *DRD2* by *SLC6A3* interaction effect was also significant in the subset of Caucasian subjects (p=0.03), but not in African Americans (p=0.23).

Association of Genotypes with Smoking History in Smokers. Among smokers, we also examined the associations of *SLC6A3* and *DRD2* genotypes with quitting history and age at smoking initiation. Those with the *SLC6A3* 9 genotype reported having quit for a significantly longer period of time than those with other genotypes (480 $\pm$ 114 days versus. 233 $\pm$ 48 days, p=0.05). This effect remained significant in a linear regression model controlling for alcohol use (F=4.46, p=0.03). There were no differences in age at initiation of smoking or in the number of cigarettes smoked per day between smokers with and without the *SLC6A3* 9 genotype. *DRD2* genotype was not associated with any of these smoking history variables and no *SLC6A3* by *DRD2* interactions were detected for these variables.

Discussion

This study provides the first evidence that *SLC6A3* 9 genotype may reduce the risk for cigarette smoking. In addition, smokers who have at least one 9-repeat allele reported having quit smoking for a longer period of time, suggesting a lower level of nicotine dependence. Thus, *SLC6A3* may influence both initiation and persistence of smoking. Moreover, the effect of *SLC6A3* on risk for smoking appears to be strongly modified by *DRD2* genotype.

Based upon the epidemiological evidence, a biological hypothesis for these findings can be formulated. Since nicotine stimulates brain reward centers via dopaminergic pathways, previous studies of disorders relating to either increased or decreased dopamine are relevant. First, dopamine transporter, the protein product of *SLC6A3*, has been associated with Parkinson's disease (22). This condition is related to decreased synaptic dopamine, and nicotine has been shown to have protective effects (23). Second, the *SLC6A3* 9-repeat allele has been associated

with cocaine-induced paranoia (20), a state attributed to excess dopamine. The *SLC6A3* 10 allele, the other common allele, has been associated with attention deficit disorder (24) and Tourette's syndrome (25), two conditions attributed to insufficient synaptic dopamine. This epidemiological evidence suggests that the *SLC6A3* 9 allele might be associated with increased synaptic dopamine (and the 10 allele with decreased dopamine). Individuals with *SLC6A3* genotypes containing the 9 allele should therefore have less need to use nicotine in order to stimulate dopamine transmission. Moreover, since the *DRD2* A1 allele has been mechanistically related to a reduced density of D_2 receptors (19), this protective effect of the *SLC6A3* 9 allele (and increased synaptic dopamine) on smoking behavior may be especially pronounced in persons who lack the *DRD2* A1 allele (and have normal receptor density). In other words, the availability of synaptic dopamine may only decrease the need for nicotine if there are sufficient receptors for normal dopamine transmission. It should be noted, however, that the lack of a known qualitative or quantitative biological effect of the *SLC6A3* VNTR polymorphism makes these conclusions tentative.

Limitations. Association studies examining candidate genes can provide more useful information than genetic linkage studies when studying common traits that are influenced by multiple genes with small effects (26). However, there are some limitations to studies such as this (27). For example, it is possible that these associations of *SLC6A3* genotype with smoking are due to the effects of a third variable, such as body mass index alcohol intake, or psychotropic medications. In the study reported here, these variables are unlikely confounders, because the main and interacting effects of *SLC6A3* and *DRD2* were not altered when these variables and

other sociodemographic covariates were controlled in multivariate models. Another potential source of bias is ethnic differences in allelic frequencies that relate to smoking behavior between smokers and nonsmokers. Our large sample size and the fact that smokers and nonsmokers were race-matched and recruited through similar mechanisms makes this source of bias less likely. However, it should also be noted that subjects responding to newspaper advertisements for smoking cessation or nonsmoking volunteers might not be representative of the general population.

Implications. To understand the role of dopaminergic genes in smoking behavior, it will be necessary to examine the interplay of the multiple genes involved in synthesis, release, receptor interaction, and re-uptake. Other neurotransmitter pathways, nicotine metabolism, and environment factors (e.g., exposure to peer smoking, tobacco advertising) will also play a role. A better understanding of genetic, neuropharmacologic, and environmental risk factors can lead to the development of improved prevention and treatment strategies tailored to the needs of individual smokers. For example, smokers with a genetic propensity to smoking may respond better to pharmacologic therapy involving nicotine replacement and/or psychotropic medications (2,28,29), whereas such approaches may be less effective for persons who have smoking habits that are more behaviorally-determined.

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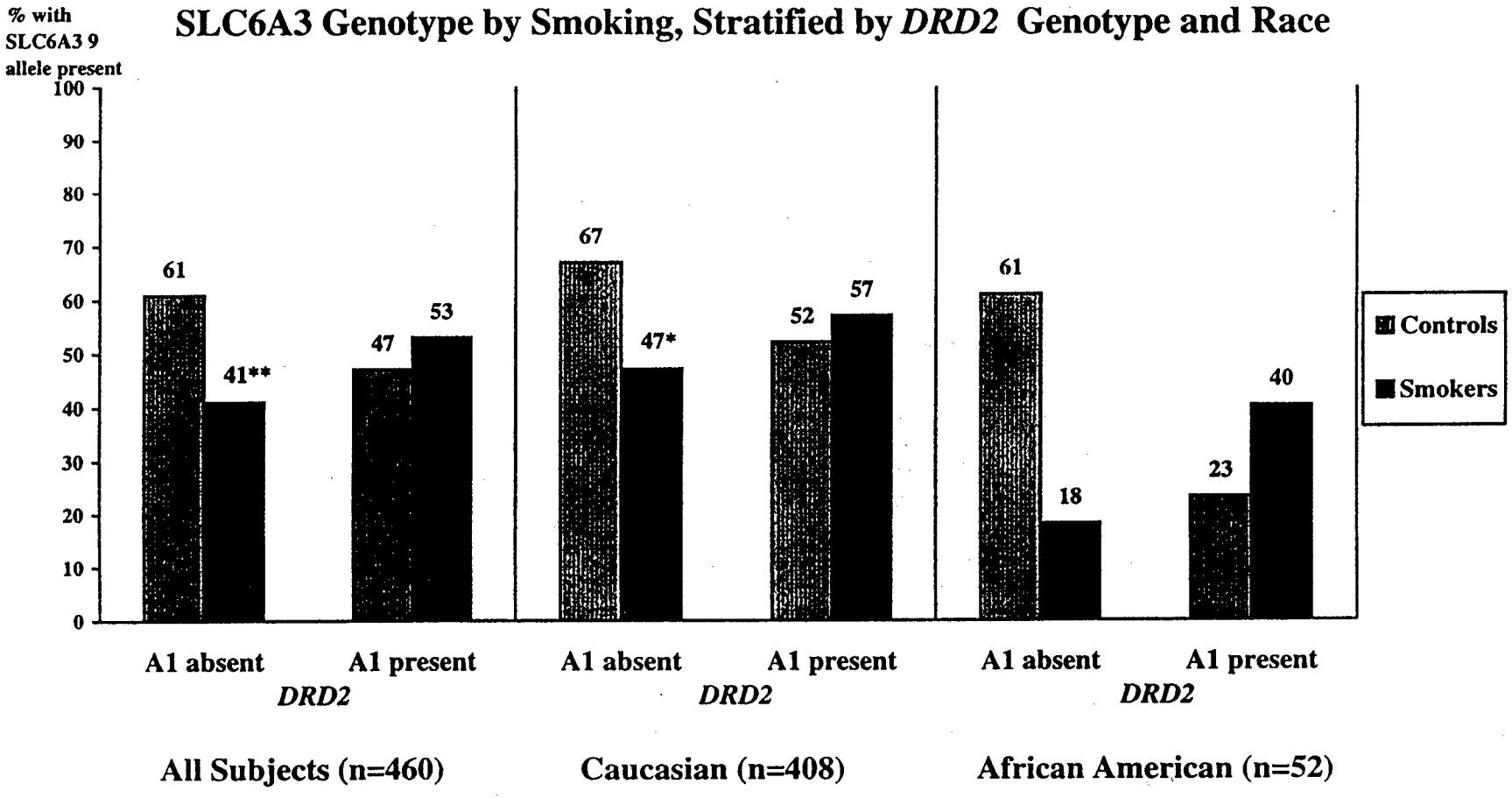
Table 1. Association of *SLC6A3* Genotype and *DRD2* Genotype with Smoking

Sample	Group	*SLC6A3* Genotype		*DRD2* Genotype	
		9/9 + 9/*	* / *	A1/A1+A1/A2	A2/A2
		n (%)	n (%)	n (%)	n (%)
All Subjects (n = 460)	Smokers	106 (46.1)	124 (53.9)	90 (39.1)	140 (60.9)
	Nonsmokers	129 (56.1)	101 (43.9)	79 (34.3)	151 (65.7)
		$\chi^2 = 4.60$, p=0.03		$\chi^2 = 1.13$, p=0.29	
Caucasians (n = 408)	Smokers	98 (48.0)	106 (52.0)	75 (36.8)	129 (63.2)
	Nonsmokers	120 (58.8)	84 (41.2)	66 (32.3)	138 (67.6)
		$\chi^2 = 4.77$, p=0.03		$\chi^2 = 0.88$, p=0.35	
African Americans (n = 52)	Smokers	8 (30.8)	18 (69.1)	15 (57.7)	11 (42.3)
	Nonsmokers	9 (34.6)	17 (65.4)	13 (50.0)	13 (50.0)
		$\chi^2 = 0.09$, p=0.77		$\chi^2 = 0.31$, p=0.58	

* denotes *SLC6A3* allele other than 9

Figure 1. *SLC6A3* Genotype by Smoking, Stratified by *DRD2* Genotype and Race

Figure 1.



* p value for chi square = .002; **p value = .001

APPENDIX Q – Dopamine D4 receptors and the risk of cigarette smoking in African-Americans and Caucasians

Shields, P. G., Lerman, C., Audrain, J., Bowman, E. D., Main, D., Boyd, N. R. and Caporaso, N. E.: Dopamine D4 receptors and the risk of cigarette smoking in African-Americans and Caucasians. Cancer Epidemiol. Biomarkers Prev., 7: 453-458, 1998.

Dopamine D4 Receptors and the Risk of Cigarette Smoking in African-Americans and Caucasians[1]

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Abstract

An understanding of why people smoke cigarettes can have an important impact on smoking prevention and cessation. People smoke cigarettes to maintain nicotine levels in the body, and nicotine has been implicated in the stimulation of brain reward mechanisms via central neuronal dopaminergic pathways. In this study, we evaluated the association of smoking and smoking cessation with a dopamine D4 receptor 48-bp variable nucleotide tandem repeat polymorphism in which the seven-repeat allele (*D4.7*) reduces dopamine affinity. Smokers (n = 283) and nonsmokers (n = 192) were recruited through local media for a case-control study of smoking. After giving informed consent and answering a behavioral questionnaire, smokers underwent a single minimal-contact session of smoking cessation counseling and then were followed for up to 1 year. The frequency of the dopamine D4 receptor genetic polymorphism using PCR was determined, and individuals were classified by the number of repeat alleles (two to five repeats as *S* and six to eight repeats as *L*). Persons with those genotypes including only *S* alleles (homozygote *S/S*) were compared with those with at least one *L* allele (heterozygote *S/L* and homozygote *L/L*). χ^2 tests of association, Fisher's exact test, and Student's t test were used. *P*s were two-tailed. The data show that African-Americans (n = 72) who had at least one *L* allele had a higher risk of smoking (odds ratio, 7.7; 95% confidence interval, 1.5–39.9; P = 0.006), shorter time to the first cigarette in the morning (P = 0.03), and earlier age at smoking initiation (P = 0.09) compared with homozygote *S/S* genotypes. After smoking cessation counseling, none of the African-American smokers with an *L* allele were abstinent at 2 months, compared with 35% of the smokers who were homozygote *S/S* (P = 0.02). The analysis of Caucasians (n = 403) did not suggest a similar smoking risk for the D4 genotypes (odds ratio, 1.0; 95% confidence interval, 0.6–1.6; P = 0.90), or smoking cessation (P = 0.75). Although the number of African-Americans is small, this study is consistent with the hypothesis that the *L* alleles increase the risk of smoking because these individuals are prone to use nicotine to stimulate synaptic dopamine transmission. If replicated, the data indicate that a single minimal-contact session of cessation counseling, similar to what is typically provided in primary care physician offices, is ineffective in African-American smokers who have at least one *L* allele. The finding of an effect for these polymorphic loci in African-Americans, but not Caucasians, suggests that the variable nucleotide tandem repeat studied here is a marker for another polymorphic site in African-Americans, but not in Caucasians.

Introduction

Tobacco smoking is a major cause of morbidity and mortality in the United States and other industrialized countries. To reduce the medical consequences of smoking, better means to prevent the initiation of tobacco smoking and addiction and to foster smoking cessation are needed. Although factors such as family history, peer pressure, advertising, and the cost of cigarettes may contribute to smoking, the most significant determinant is nicotine dependence (1). Host susceptibilities may play a role in nicotine dependence through interindividual variation in nicotine metabolism or through neurobehavioral factors that relate to the reinforcing value of nicotine. The former may dictate the initial pharmacological reactions to nicotine and how much smoking is needed to maintain nicotine levels (1, 2), whereas the latter may affect why people need to maintain nicotine levels.

Nicotine has a "rewarding" property that serves to reinforce drug-seeking behavior (1–3). Nicotine stimulates central nicotinic acetylcholine receptors, which are up-regulated and desensitized simultaneously by chronic exposure. These receptors stimulate the secretion of dopamine into the neuronal synapse; the dopamine then stimulates postsynaptic dopamine receptors and thereby satisfies craving. The relationship of nicotine to the dopaminergic system is well established, as is the effect of nicotine on psychiatric illness (4). The stimulation of the dopaminergic system is not solely dependent on nicotine, however; for example, cocaine, amphetamine, and food also affect dopamine pathways (5, 6).

We have hypothesized that interindividual variation for dopamine pathways and the reward mechanism might alter the risk of smoking. To examine this hypothesis, we have studied polymorphisms in genes that govern synaptic dopamine levels through active reuptake by the dopamine transporter and in

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dopamine receptors. These results have provided evidence that the risk of smoking is related to a genetic polymorphism in the dopamine reuptake transporter gene and that there is an interaction with a dopamine D2 receptor polymorphism (7). Other studies also have suggested that the same genetic polymorphism in the dopamine D2 receptor is related to smoking (8, 9).

Postsynaptic dopamine receptors (10, 11) can be classified as D1-like or D2-like. The dopamine D4 receptor (12) is an example of the latter group. There are differences between the dopamine D2 and D4 receptors, however, such as in dopamine affinity (greater for the D4 receptor in the low-affinity receptor state) and in the levels of protein expression (10). There also is a difference for the binding of the dopamine agonist clozapine, which is an order of magnitude higher for the D4 receptor compared with the D2 receptor (12–15). Clozapine is used for the treatment of schizophrenia, a disease that has a hypothesized underlying defect in the dopamine reward system. The D4 receptor affects G protein-mediated functional coupling (16) and has been reported to be increased in schizophrenic patients (13, 16), although not consistently (17).

There is an imperfect 48-bp VNTR[3] polymorphism involving 18 amino acids in the third exon of the dopamine D4 receptor, which codes for a proline-rich protein domain in the third cytoplasmic loop (18, 19). The seven-repeat allele (*D4.7*) has been associated with increased competition for [^{3}H]spiperone binding in initial studies (18), which accounts for increased clozapine binding. A subsequent *in vitro* study using transiently expressed COS-7 cells found that the cyclic AMP effect of dopamine for *D4.7* was reduced about 2-fold, although the overall effects of the polymorphism were considered small (20). Nonetheless, the data suggest that *D4.7* is associated with a blunted response to dopamine, although a clinical effect from this polymorphism on the response to clozapine therapy in schizophrenia could not be shown (21).

If *D4.7* has a blunted response to dopamine *in vivo* and given that the effect of nicotine is to increase synaptic dopamine, it is plausible that people with the *D4.7* allele might have more nicotine dependence, resulting in a greater risk of smoking and less ability to quit smoking after cessation counseling. To test this hypothesis, we studied the dopamine D4 receptor polymorphism in smokers and nonsmokers and then examined the ability of the smokers to abstain from smoking after minimal-contact behavioral cessation treatment.

Subjects and Methods

Study Subjects. Smokers (n = 283), ages 18 and over, seeking a free smoking-cessation program were recruited through media advertising (newspapers and flyers) in the Washington, DC and Philadelphia areas. Smokers were defined as smoking at least five cigarettes per day for at least 1 year. Their mean age was 42.0 (±16.7). Controls (n = 192) also were recruited through the newspaper advertisements and flyers and were defined as having smoked less than 100 cigarettes in their lifetime. Their mean age was 43 (±11.0). The exclusion criteria for the study were age <18, a personal history of cancer, concurrent treatment for drug or alcohol addiction, or the presence of a psychiatric disorder that precluded informed consent.

Procedures. During a visit to the clinic, subjects completed an informed consent form and a questionnaire assessing demographics and smoking history. Subjects then received a single minimal-contact session (1 h) of behavioral smoking-cessation counseling and self-help materials (22). They were then followed-up for 1 year after the counseling to assess self reports of quitting. The outcome measure was a 7-day point prevalence of smoking (persons who self-reported smoking within 7 previous days) at 2 months and 12 months after smoking cessation treatment. All subjects donated blood for genetic analysis. Subjects were blinded to their dopamine D4 genotype status; therefore, knowledge of these results would not influence the smoking cessation outcome.

Table 1 Allele frequency for smokers and nonsmokers by race

Allele	Nonsmokers (%)		Smokers (%)	
	Caucasians	African-Americans	Caucasians	African-Americans
D4.2	44 (13)	11 (23)	49 (10)	6 (6)
D4.3	15 (5)	1 (2)	23 (5)	2 (2)
D4.4	236 (70)	31 (65)	331 (70)	61 (64)
D4.5	2 (1)	3 (6)	4 (0.8)	2 (2)
D4.6	0 (0)	0 (0)	1 (0.2)	0 (0)
D4.7	37 (11)	2 (4)	59 (13)	24 (25)
D4.8	2 (1)	0 (0)	3 (0.6)	1 (1)
Total	336	48	470	96

Dopamine D4 Receptor Genotyping. DNA was extracted from whole blood buffy coats using standard phenol extraction methods. PCR for the dopamine D4 48-bp VNTR in exon 3 was performed based on the method of George *et al.* (23). Briefly, genomic DNA (25 ng) was amplified using 20 pmol of primers (5′-CTG CGG GTC TGC GGT GGA GTC TGG-3′ and 5′-GCT CAT GCT GCT GCT CTA CTG GGC-3′) in 5% DMSO, 10% glycerol, buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.0 mM $MgCl_2$], 0.63 unit of Amplitaq DNA polymerase (Perkin-Elmer, Norwalk, CT), and 1.87 mM 2′-deoxynucleoside-5′-triphosphate (Pharmacia, Piscataway, NJ) in a 25-μl volume. The primers were synthesized using a Beckman Oligo1000 DNA synthesizer (Fullerton, CA). The PCR reaction had an initial melting temperature of 95°C (4 min), followed by 30 cycles of melting (95°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 1 min). An extension period at 72°C (4 min) followed. The PCR reactions were performed using a Stratagene (La Jolla, CA) Robocycler Gradient 96 apparatus. Fragments ranging from 270 to 570 bp (two to eight repeats) were resolved by agarose gel electrophoresis [Nusieve GTG (Life Technologies, Inc., Gaithersburg, MD) and agarose, 2:1 (w/v), 3% total] and detected with ethidium bromide staining. The assay was validated by confirming polymorphic Mendelian inheritance patterns in seven human family cell lines (n = 134 family members) encompassing three generations (data not shown; samples were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Coriell Institute, Camden, NJ). Genotyping results were read by two independent investigators, and genotyping for 20% of the subjects was repeated for quality control. The investigators were blinded to each other's interpretations and to the smoking status of the subjects.

Statistical Analysis. The dopamine D4 receptor was classified as reported previously (24), based on the number of 48-bp repeats in exon 3, and by genotypes representing the overall length (*S* consisted of 2, 3, 4, or 5 repeats, and *L* alleles consisted of 6, 7, or 8 repeats). Most (89%) of the *L* alleles were the seven repeats (*D4.7*; Table 1), so that classification by *D4.7* prevalence alone would not appreciably alter the research results. Associations of alleles and genotypes with current smok-

[3] The abbreviations used are: VNTR, variable nucleotide tandem repeat; OR, odds ratio; CI, confidence interval.

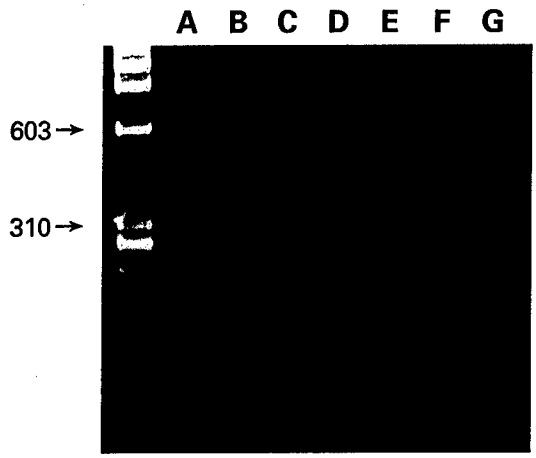

Fig. 1. Representative genotypes for the dopamine D4 48-bp VNTR polymorphism. The following genotypes are shown: 2/7 (*A*), 2/3 (*B*), 2/4 (*C*), 3/4 (*D*), 4/4 (*E*), 4/7 (*F*), and 4/8 (*G*).

ing and categorical smoking variables [age at smoking initiation (more than or less than 16 years) and time to the first cigarette in the morning (more than or less than 30 min)] and 7-day point prevalence (persons who self-reported smoking within 7 previous days) for 2 and 12 months of follow-up were examined using χ^2 tests of association, except when the Fisher's exact test was used as indicated. When examining continuous variables (*e.g.*, smoking rate), Student's *t* test or ANOVA was used. ORs and their 95% CIs were calculated by unconditional logistic regression using SAS software (Statistical Analysis System, Cary, NC). All *P*s were two-tailed.

Results

There were 283 smokers and 192 controls who were genotyped for the D4 genetic polymorphism (Fig. 1). The allelic frequencies for smokers and nonsmokers by race are shown in Table 1, in which *D4.7* is shown to be more common in African-Americans. There was a statistically significant difference in the prevalence of *L* and *S* alleles between Caucasians and African-Americans for smokers ($P = 0.004$), but not for nonsmokers ($P = 0.18$). The frequency of genotypes were in Hardy-Weinberg equilibrium for smokers and nonsmokers when examined separately by race. Because of the differences in allelic frequency for Caucasians and African-Americans, subsequent analyses are presented separately for each racial group.

For African-Americans, there was a significant association of the homozygote *S/S versus* heterozygote *S/L versus* homozygote *L/L* genotypes with smoking (Table 2; $P = 0.02$, Fisher's exact test), although the number of study subjects was small ($n = 71$). The difference for the African-Americans with the *S/S* genotypes compared with the *S/L* genotypes or *L/L* genotypes was highly significant ($\chi^2 = 7.56$, $P = 0.006$). The OR for the risk of smoking in African-Americans with at least one *L* allele was 7.7 (95% CI, 1.5–39.9). In addition, African-American smokers who had the *S/L* or *L/L* genotypes were more likely to smoke within 30 min of waking (95% *versus* 69%, $\chi^2 = 4.62$, $P = 0.03$) and to have started smoking before the age of 16 years (47% *versus* 24%, $\chi^2 = 2.84$, $P = 0.09$). There was no association with the number of cigarettes smoked/day using Student's *t* test (Table 3).

Tables 3 and 4 show the prospective data for smoking abstinence after minimal-contact behavioral smoking cessation counseling. Overall, 23 and 17% of African-Americans reported quitting at 2 and 12 months, respectively. When analyzed by genotype, none of the African Americans with the *S/L* or *L/L* genotypes reported having quit smoking at 2 months of follow-up, whereas 35% of the persons with the *S/S* genotype reported quitting ($P = 0.02$). At 12 months, the data were identical, but because of the smaller number of subjects, the result was not statistically significant ($P = 0.29$). The odds of resuming smoking for the *S/L* or *L/L* genotypes could not be estimated because the proportion of abstainers was 0%.

Table 2 Association of dopamine *D4* receptor genotypes and smoking

Genotype[a]	Nonsmoker (%)	Smoker (%)	*P*
Caucasians			
S/S	132 (79)	183 (78)	0.28[b,c]
S/L	33 (19)	41 (18)	0.90[d]
L/L	3 (2)	11 (4)	
African-Americans			
S/S	22 (92)	29 (60)	0.02[b,c]
S/L	2 (8)	13 (27)	0.006[d]
L/L	0 (0)	6 (13)	

[a] *S* = *D4.2, D4.3, D4.4,* or *D4.5; L* = *D4.6, D4.7,* or *D4.8.*
[b] Fisher's exact test.
[c] *P* for *S/S versus S/L versus L/L.*
[d] *P* for *S/S versus S/L* or *L/L.*

For Caucasians, the presence of the homozygote *L/L versus* heterozygote *S/L versus* homozygote *S/S* genotypes was not associated with smoking (Table 2). The OR for the risk of smoking in Caucasians with at least one *L* allele was 1.0 (95% CI, 0.6–1.6). There was no association for the *S/L* or *L/L* genotypes with the number of cigarettes smoked/day (Student's $t = 0.78$, $P = 0.43$), age when smoking began ($\chi^2 = 0.00$, $P = 0.94$), or time to the first cigarette in the morning ($\chi^2 = 1.96$, $P = 0.16$; Table 3). There was no difference for the *S/S versus S/L* or *L/L* for the likelihood of having quit smoking at the 2-month ($P = 0.75$) or 12-month ($P = 0.08$) follow-up (Table 4). Overall, only 15 and 17% could quit smoking at 2 and 12 months, respectively. For persons with the *S/S* genotype, 15% could abstain after cessation therapy at the 2- and 12-month follow-ups, whereas 16 and 27% of the *S/L* and *L/L* individuals continued to abstain at 2 and 12 months, respectively. The OR for continuing to smoke and having the *S/L* or *L/L* genotypes was 0.85 (95% CI, 0.35–2.11) at 2 months and 0.45 (95% CI, 0.19–1.09) at 12 months.

Discussion

This study indicates that the 48-bp VNTR polymorphism in the third exon of the dopamine D4 receptor gene, specifically persons with *D4.6, D4.7,* or *D4.8,* may be a risk factor for smoking in African-Americans but not in Caucasians. The data are internally consistent for African-Americans in that those persons with the *S/L* or *L/L* genotypes had a higher risk of smoking at entry into the study, a shorter time to the first cigarette in the morning, a lower age at smoking initiation, and an increased inability to quit after counseling. However, the number of African-Americans in this study is small. Given that the *D4.7* repeat VNTR for the dopamine D4 receptor alters the structure of the receptor, affects dopamine agonistic binding for clozapine, and reduces the effects of the receptor 2-fold (18, 20, 25), the relationships of this genetic polymorphism to the risk of smoking might be caused by a need for nicotine to increase synaptic dopamine. The finding that the dopamine D4 VNTR polymorphism is a risk factor for smoking in African-Ameri-

Table 3 Characteristics of smokers by dopamine D4 receptor genotypes

	S/S		*S/L*		*L/L*	
	Mean[a]	Range	Mean[a]	Range	Mean[a]	Range
Caucasians						
Longest time quit smoking (days)	348 (795)	0–5478	386 (731)	0–2922	278 (643)	0–2191
Age started to smoke	17 (4)	8–34	17 (5)	8–32	17 (3)	15–16
Cigarettes/day in the last week	23 (10)	3–50	21 (12)	4–40	23 (12)	6–50
Number of quit attempts lasting 24 h at follow-up						
4-months	2 (1)	0–6	3 (4)	1–15	3 (4)	1–12
12 months	3 (3)	0–12	4 (4)	1–20	2 (1)	1–4
African-Americans						
Longest time quit smoking (days)	184 (322)	0–1095	205 (406)	1–1095	1,1171 (2,412)	0–5478
Age started to smoke	17 (3)	12–29	16 (4)	12–24	18 (3)	14–23
Cigarettes/day in the last week	18 (9)	3–43	15 (9)	5–40	13 (6)	7–20
Number of quit attempts at follow-up						
4-month	3 (2)	1–9	2 (1)	1–3	1 (11)	1–2
12-month	4 (5)	1–20	3 (2)	1–5	1 (1)	1–2

[a] Numbers in parentheses indicate standard deviation.

Table 4 Association of dopamine D4 receptor genotypes and smoking cessation at 2- and 12-months

Genotype[a]	Abstain			Smoking			*P*
	No.	Row %	Column %	No.	Row %	Column %	
2-month follow-up							
Caucasians							
S/S	23	15	77	134	85	79	0.78[b,c]
S/L	5	15	17	28	85	16	0.75[d]
L/L	2	22	7	7	78	4	
Total	30	15		169	85		
African-Americans							
S/S	9	35	100	17	65	55	0.06[b,c]
S/L	0	0	0	8	100	26	0.02[d]
L/L	0	0	0	6	100	19	
Total	9	23		31	77		
12-month follow-up							
Caucasians							
S/S	18	15	64	106	85	80	0.12[b,c]
S/L	7	24	25	22	76	17	0.08[d]
L/L	3	38	11	5	62	4	
Total	28	17		133	83		
African-Americans							
S/S	5	24	100	16	76	0.6	0.43[b,c]
S/L	0	0	0	4	100	16	0.29[d]
L/L	0	0	0	5	100	20	
Total	5	17		25	83		

[a] *S* = *D4.2, D4.3, D4.4,* or *D4.5; L* = *D4.6, D4.7,* or *D4.8.*
[b] Fisher's exact test.
[c] *P* for *S/S versus S/L versus L/L.*
[d] *P* for *S/S versus S/L* or *L/L.*

cans but not in Caucasians suggests that VNTR is not functionally related to such a factor. Also, the experimental relationship of *D4.7* to dopamine binding seems to be less than what would be predicted for the findings in this study. It is more likely that in African-Americans, *D4.7* is linked to another polymorphic site with the dopamine D4 receptor gene, or another gene, that governs the smoking risk variability, whereas this site is not polymorphic in Caucasians. Indeed, there are other known genetic polymorphisms located within the dopamine D4 receptor (26, 27), one of which is present only in African-Americans (26), is located in exon 3, and accounts for altered dopamine binding (28). However, the relationship of this latter polymorphism to *D4.7* has not yet been established.

The relationship of the dopamine D4 receptor exon 3 VNTR polymorphism to smoking has received little attention. One study (23) examined the polymorphism in relation to smoking in alcoholics and did not find an association, but alcoholism is a separate addictive disease; therefore, the results from that study may not be applicable to the findings contained herein. However, this polymorphism, and specifically *D4.7,* has been related to neurological illness and personality, specifically in two of three studies investigating novelty-seeking behavior

(24, 29, 30), a personality pattern associated with smoking. *D4.7* also has been seen with attention deficit disorder (31) and Tourette's syndrome families (32), and although there was no evidence for an association with bipolar affective disorder (33), there was a reported trend in one of two studies of schizophrenia (34, 35).

The associations of the D4 *S/L* and *L/L* genotypes with smoking practice and cessation outcomes in African-Americans and the fact that these genotypes are significantly more common in African-Americans than Caucasians are consistent with a growing body of literature on race differences in smoking. For instance, the population prevalence of smoking is significantly higher in African-American men than in Caucasian men (34% *versus* 28%; Ref. 36), and African-Americans have higher rates of smoking-related morbidity and mortality (37, 38). The higher smoking-related health hazards seem inconsistent with the findings that African-Americans tend to initiate smoking at a later age and smoke less than Caucasians (39, 40), although smoking initiation is complex because it is dependent on exposure to tobacco products (*e.g.,* by way of peer pressure and advertising). However, African-Americans tend to smoke cigarettes with a higher tar and nicotine content (41–43) and report higher levels of nicotine dependence than Caucasians (44). Our results suggest that African-Americans with the *D4 S/L* or *L/L* genotypes may be especially predisposed to smoke and to become nicotine dependent.

Although African-Americans attempt to quit smoking as often as Caucasians (43, 45, 46), they are less likely to succeed (46, 47). In the present study, quit rates for African-Americans and Caucasians were 23 and 15% at 2 months and 17 and 17% at 12 months, respectively. This is consistent with previous studies of minimal-contact smoking cessation treatments (48, 49). Our preliminary results suggest that African-Americans who have the *D4 S/L* or *L/L* genotypes may have an especially difficult time abstaining from smoking at 2 months, because none of these African-Americans could quit. Because of the higher level of nicotine dependence in African-Americans (44) and a possible genetic predisposition to become dependent on nicotine, African-American smokers may be especially good candidates for treatments that use nicotine replacement (50, 51). Furthermore, the dopamine D4 receptor polymorphism in African-Americans may be useful in discerning who would be better candidates for minimal-contact behavioral therapy (*S/S* genotypes) and who should have other therapies, such as nicotine replacement or psychotropic medications (*S/L* or *L/L* genotypes).

There are several limitations in this study:

(*a*) Smokers recruited through the media may not represent smokers in the general population, especially when the enrollees are recruited for a smoking cessation program. Typically, these are persons who are the most addicted in the population because they have been unable to quit on their own.

(*b*) Because of the low gene frequency, the number of subjects with the *L* alleles is very small. Thus, there is limited statistical power to detect positive associations. Separately, there is a possibility that there may be an association of smoking with specific haplotypes that we did not study. Although we have examined the associations by genotypes characterized by *L* or *S* alleles, in actuality there are more than 25 different haplotypes that code for 18 different predicted amino acid sequences (52). At this time, the functional effects of these haplotypes on clozapine binding or receptor structure are not known, and the effects of these haplotypes can be investigated only in much larger studies to have sufficient statistical power.

(*c*) The distribution of dopamine *D4* alleles that we found in either the smokers or the nonsmokers may not be representative of the African-American population. However, to date, there are no published frequencies for these alleles in the United States; a survey from several parts of Africa indicate that the *D4.7* allele frequency ranges from 0 to 0.19 (53).

(*d*) Although we verified abstinence with biochemical markers such as cotinine, this was done only on a subset. It is possible that the African-American reports of abstinence were incorrect, although there is no reason, *a priori,* to believe that this would occur in relation to the dopamine D4 genetic polymorphism.

Despite the limitations noted above, this study is the first to identify a genetic polymorphism associated with smoking practices and the ability to quit in African-Americans. It suggests that there may be differences in the effects of genetic susceptibilities by race. Additional studies are needed to corroborate the findings presented here. Nonetheless, a better understanding of the genetic determinants of smoking could enhance present efforts to prevent and treat nicotine dependence in this population. Specifically, if additional studies identify the same findings, by using these approaches, the dopamine D4 48-bp VNTR polymorphism might be used to focus individual smoking cessation therapy. Although the single counseling session used in this study would be considered to be inadequate therapy, it is similar to what typically occurs in a primary care physician's office. In this case, for African-Americans who have the *S/S* genotype, a single minimal-contact behavioral smoking cessation session might be successful. However, for African-Americans with the *S/L* or *L/L* genotypes, this would be inadequate.

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APPENDIX R – Lack of association of tyrosine hydroxylase genetic polymorphism with cigarette smoking

Lerman, C., Shields, P. G., Main, D., Audrain, J., Roth, J., Boyd, N. R. and Caporaso, N. E.: Lack of association of tyrosine hydroxylase genetic polymorphism with cigarette smoking. Pharmacogenetics. 7: 521-524, 1998.

Pharmacogenetics (1997) **7**, 521–524

Short communication

Lack of association of tyrosine hydroxylase genetic polymorphism with cigarette smoking

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Cigarette smoking is a major risk factor for cancer, atherosclerotic vascular disease, respiratory illness and early mortality. Twin studies (Carmelli *et al.*, 1992) have indicated that inherited factors account for as much as 50% of the variance in cigarette smoking practices. Genes involved in dopamine regulation are plausible candidates for these genetic effects because the reinforcing properties of nicotine have been attributed, in part, to its effects on dopamine transmission (Henningfield *et al.*, 1995). Indeed, preliminary studies have reported associations of cigarette smoking with genes governing synaptic dopamine receptor function (Comings *et al.*, 1996).

While not yet tested, several lines of evidence suggest that the tyrosine hydroxylase (TH) gene may play a role in cigarette smoking. Tyrosine hydroxylase converts phenylalanine to dopamine and is a rate limiting enzyme involved in the synthesis of catecholamines. The tyrosine hydroxylase gene is located on chromosome 11p15. It comprises a single gene that results in four different protein isoforms present in the brain, which are generated by alternative RNA processing in intron 1 (O'Malley *et al.*, 1987). Nicotine has been shown to increase the expression of tyrosine hydroxylase in cultured cell models (Hiremagalur *et al.*, 1993). Transgenic mice that overexpress TH are less sensitive to nicotine (Nabeshima *et al.*, 1994). Furthermore, disregulation of TH appears to be important in Parkinson's disease (Javoy-Agid *et al.*, 1990), a condition for which nicotine has protective effects (Newhouse & Hughes, 1991). Tetranucleotide repeat polymorphisms in the *TH* gene have been associated with bipolar disorder (Todd & O'Malley, 1989), but this was not replicated in subsequent studies (Körner *et al.*, 1994). The possibility of an association with bipolar disease is of interest because of the well established link between depression and smoking (Lerman *et al.*, 1996).

The aim of the present study was to examine the association of polymorphisms in the *TH* gene with smoking in Caucasians and African Americans. Smokers ($n = 315$) who reported smoking at least 5 cigarettes a day for at least 1 year were recruited through newspaper advertisements and flyers in the metropolitan Washington and Philadelphia areas for a free smoking cessation program. Non-smoking control individuals ($n = 232$) who reported having smoked fewer than 100 cigarettes in their lifetimes were recruited through a similar protocol. Exclusion criteria were: aged under 18 years; a personal history of cancer; undergoing treatment for drug or alcohol addiction, or presence of a psychiatric disorder that precluded informed consent.

During a visit to the clinic, subjects completed a questionnaire assessing demographic factors and smoking

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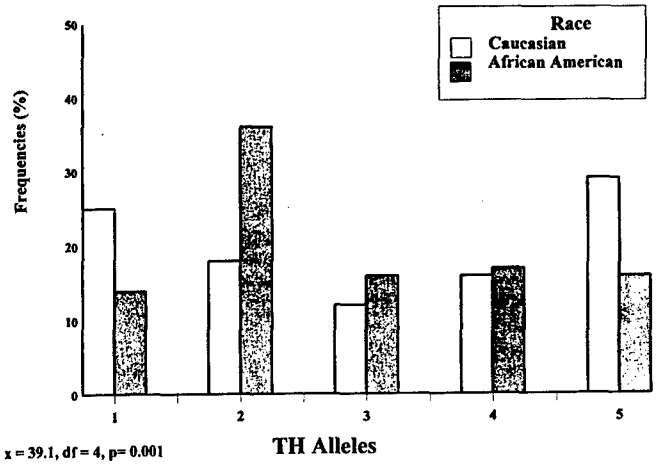

Fig. 1. TH Alleles Frequencies for Caucasians and African Americans

history and donated blood for analysis. DNA was extracted from whole blood using standard phenol extraction methods. PCR for the tyrosine hydroxylase tetranucleotide repeat polymorphism, which begins at base pair 1170 in intron 1, was performed using primers reported by Polymeropoulos (1991). These primers (40 pmol each per 100 µl reaction mixture) were mixed with genomic DNA (25 ng), buffer (tris-HCl [67 µM, pH 8.8], $MgCl_2$ [2.0 µM], NH4SO4 [16.6 µM], β-mercaptoethanol [50 µM], EDTA [6.8 µM], and bovine serum albumin [80 ng ml^{-1}], Amplitaq DNA polymerase (5 U per 100 µl; Perkin Elmer, Norwalk, CT, USA) and 2′-deoxynucleosides-5′-triphosphates (150 µM; Pharmacia, Piscataway, NJ, USA) in a 100 µl total reaction mixture volume. The PCR reaction had an initial melting temperature of 94 °C (5 min), followed by 35 cycles of melting (94 °C; 1 min), annealing (65 °C; 1 min) and extension (72 °C; 1 min). An extension period at 72 °C (7 min) followed. This reaction yielded fragments ranging from 245 bp to 261 bp. The reaction mixture was then subjected to *Hin* FI restriction endonuclease digestion, in order to better resolve the tetranucleotide fragments. This digestion yielded constant fragments of 147 bp and 33 bp, and variable fragments ranging in size from 65 to 81 bp. The fragment lengths were then determined using polyacrylamide gel electrophoresis (10%). The assay was validated by confirming polymorphic Mendelian inheritance patterns in seven human family cell lines (n = 134 family members), encompassing three generations (data not shown; samples were obtained from NIGMS Human Genetic Mutant Cell Repository, Coriell Institute, Camden, NJ, USA). For all assays, 10% of samples were repeated for quality control. All gels were read independently by two investigators who were blind to case status.

As in previous studies (Körner *et al.*, 1994), the frequencies of the five alleles of the *TH* gene were examined for associations using chi-squared tests of association. Statistical analyses were performed separately for Caucasians and for African Americans.

The racial composition of the sample was 85% Caucasian (n = 466), and 15% African American (n = 81). The average age was 43 years (range = 18–80 years). Fifty-seven percent were female and 43% were male. Most were college graduates (59%); however, this was less likely among smokers (55%) than among non-smokers (65%) ($\chi^2 = 8.4$, $p = 0.08$). The frequencies for the five *TH* alleles for Caucasians were consistent with previous reports (Polymeropoulous *et al.*, 1991; Korner *et al.*, 1994). As shown in Fig. 1, there were significant differences between Caucasians and African Americans in allele frequencies ($\chi^2 = 39.1$, d.f. = 4, $p = 0.001$). The *TH2* allele was more common in African Americans and the *TH1* and *TH5* alleles were less common, as

Table 1. TH allele frequencies in smokers and non-smokers

	Allele numbers and frequences (%)				
	1	*2*	*3*	*4*	*5*
Caucasians[a]					
Smokers	128 (25%)	100 (19%)	59 (11%)	87 (17%)	148 (28%)
Non-smokers	101 (25%)	70 (17%)	52 (13%)	66 (16%)	121 (29%)
African Americans[b]					
Smokers	15 (14%)	40 (37%)	18 (17%)	19 (17%)	16 (15%)
Non-smokers	8 (15%)	19 (35%)	9 (17%)	8 (15%)	10 (18%)
All subjects[c]					
Smokers	143 (23%)	140 (22%)	77 (12%)	106 (17%)	164 (26%)
Non-smokers	109 (24%)	89 (19%)	61 (13%)	74 (16%)	131 (28%)

[a] $\chi^2 = 1.07$, d.f. = 4, $p = 0.90$.
[b] $\chi^2 = 0.53$, d.f. = 4, $p = 0.97$.
[c] $\chi^2 = 2.04$, d.f. = 4, $p = 0.73$.

compared to Caucasians.

The *TH* allelic frequencies in smokers and non-smokers are shown in Table 1. There were no significant differences between smokers and non-smokers within the Caucasian sample ($\chi^2 = 1.07$, d.f. = 4, $p = 0.90$), the African American sample ($\chi^2 = 0.53$, d.f. = 4, $p = 0.97$), or in the total sample ($\chi^2 = 2.04$, d.f. = 4, $p = 0.73$). These analyses were repeated stratifying by education level and, again, the results were similar. Among smokers, associations between the presence (versus absence) of each of the five *TH* alleles and daily smoking rate using Student's *t*-test were also examined. The presence of the *TH1* allele was associated with a higher smoking rate, as compared to the absence of *TH1* (23.4 ± 0.9 versus 21.1 ± 0.7, $t = 2.02$, $p = 0.04$). The presence of the *TH4* allele was associated with a lower smoking rate, compared to the absence of *TH4* (20.4 ± 1.0 versus 22.8 ± 0.7, $t = 2.0$, $p = 0.05$). No other alleles were associated with smoking rate.

In the present study, we did not find evidence of an association between alleles at the *TH* locus and the likelihood of smoking in either Caucasians or African Americans. However, given the small number of African Americans, it is premature to draw conclusions concerning this ethnic group. While associations of the TH polymorphism with smoking have not been examined previously, preliminary data support an association of polymorphisms in other dopaminergic genes with cigarette smoking. In a recent study of the polymorphic dopamine *D2* receptor gene, the prevalence of genotypes containing the *A1* allele was 26% in control individuals as compared to 49% in smokers (Comings *et al.*, 1996). This is biologically plausible because the *A1* allele has been related to a reduced density of D2 receptors (Noble *et al.*, 1991). Thus, persons with the *DRD2 A1* allele may be more likely to use nicotine to pharmacologically augment dopamine transmission. In addition, we have found differences in the prevalence of the dopamine *D4* receptor polymorphism in African American smokers compared with non-smokers (Unpublished data). The 'long' *DRD4* alleles, particularly the 7-repeat allele, have been associated with a blunted intracellular response to dopamine (Asghari *et al.*, 1995).

The discrepancy between the results for *TH* and other dopaminergic genes may reflect the fact that *TH* does not play a role in the initiation or maintenance of smoking or that the polymorphism may be nonfunctional. Alternately, the absence of differences in allele frequencies between smokers and non-smokers may be caused by limitations of genetic association studies. One potential source of bias is ethnic differences in allelic frequencies and smoking practices. It should also be noted that the recruitment of study subjects responding to newspaper advertisements for smoking cessation or non-smoking volunteers might generate a sample that is not representative of the general population. However, our data do suggest that the presence of specific alleles (*TH1* and *TH4*) may influence smoking practices among smokers. This finding needs to be replicated in another study population.

The dopaminergic system is composed of a complex set of processes involving synthesis, release, interaction with receptors and re-uptake. To understand the role of dopaminergic genes in smoking, it will be necessary to examine the interplay of the multiple genes involved in these processes. Furthermore, other neurotransmitter pathways, nicotine metabolism and environmental factors will also play a role. All of these areas need to be studied so that a better understanding of genetic heterogeneity and neuropharmacologic mechanisms can lead to the development of improved prevention and treatment strategies tailored to the needs of individual smokers.

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APPENDIX S – The role of the serotonin transporter gene in cigarette smoking

Lerman, C., Shields, P. G., Audrain, J., Main, D., Cobb, B., Boyd, N. R. and Caporaso, N.: The role of the serotonin transporter gene in cigarette smoking. Cancer Epidemiol. Biomarkers Prev., 7: 253-255, 1998.

Short Communication

The Role of the Serotonin Transporter Gene in Cigarette Smoking

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Abstract

Data from twin studies have suggested that cigarette smoking has a significant heritable component. The serotonin transporter gene (*5-HTT*) is a plausible candidate gene for smoking predisposition because of its association with psychological traits relevant to smoking behavior. The present investigation evaluated the associations of smoking practices and smoking cessation with a common polymorphism in the upstream regulatory region of *5-HTT* that is manifested as either an inserted (long) variant or a deleted (short) variant. The short variant of the polymorphism is associated with reduced transcription of the gene promoter and diminished uptake. A case-control study design (268 smokers and 230 controls) was used to evaluate the associations of *5-HTT* genotype with smoking status. Case series analysis of smokers was used to evaluate the role of *5-HTT* in age at smoking initiation, previous quitting history, current smoking rate, and 12-month quit rate following a minimal-contact smoking cessation program. There were no significant differences in the distribution of *5-HTT* genotypes in smokers as compared with nonsmokers in either Caucasians or African Americans, nor was the *5-HTT* genotype associated with the smoking outcome variables. However, the results did reveal significant racial differences in the distribution of *5-HTT* genotypes: Caucasians were significantly more likely to carry the short variant of the *5-HTT* gene than were African Americans ($P = 0.005$). These findings suggest that the *5-HTT* gene may not play a significant role in cigarette smoking practices.

Introduction

Cigarette smoking is the greatest preventable cause of cancer mortality (1), yet approximately 26% of adults in the United States continue to smoke (2). Evidence from twin studies (3) indicates that smoking has a significant heritable component. Previously, we reported on the results of a smoking case-control study that examined associations of smoking practices with polymorphic genes important in dopamine transmission. These studies were based upon evidence supporting the role of dopamine in the brain's reward mechanism (4) and suggesting that nicotine stimulates dopamine transmission (5). We found preliminary support for an association of the dopamine transporter (*SLC6A3*) gene with the likelihood of smoking, age at smoking initiation, and previous quitting history.[2] However, the tyrosine hydroxylase gene was not associated with any smoking outcomes (6).

In this study, we examined associations of a serotonin transporter (*5-HTT*) gene with smoking practices. The serotonin transporter gene is located on chromosome 17q11.2 (7), and gene transcription has been reported to be modulated by a polymorphism in its regulatory region (8). The polymorphism is a 44-bp deletion or insertion, in which the inserted variant (long) *versus* the deleted variant (short) occurs in 57 and 43% of Caucasians, respectively (9). The short variant is associated with reduced transcription, resulting in diminished *5-HTT* uptake (8).

The *5-HTT* gene is a plausible candidate gene for smoking predisposition because of its role in psychological traits relevant to smoking behavior. The *5-HTT* polymorphism has been linked with anxiety-related personality traits (9) and with depression (10, 11); however, the former finding was not replicated in a recent analysis (12). Both anxiety and depression have been linked with nicotine dependence (13, 14). Further, preliminary clinical data suggest that serotonin reuptake inhibitors, such as fluoxetine hydrochloride, may promote smoking cessation (15, 16). Of interest, smokers who are more nicotine dependent responded better to fluoxetine treatment than less dependent smokers (16). Because the short variant of *5-HTT* has been associated with reduced uptake (*i.e.*, more available serotonin), we predicted that the presence of *5-HTT* short alleles would be protective for smoking (*i.e.*, associated with a lower likelihood of being a smoker).

In the analysis reported here, we used a case-control study design to evaluate the association of smoking practices with the *5-HTT* polymorphism. A case-series analysis of smokers was performed to examine associations of *5-HTT* with age at smoking initiation, previous quitting history, current smoking rate, and 12-month cessation rates following a minimal-contact smoking cessation treatment program.

Subjects and Methods

Subjects. Smokers ($n = 268$) who reported smoking at least 5 cigarettes/day for at least 1 year were recruited through varied

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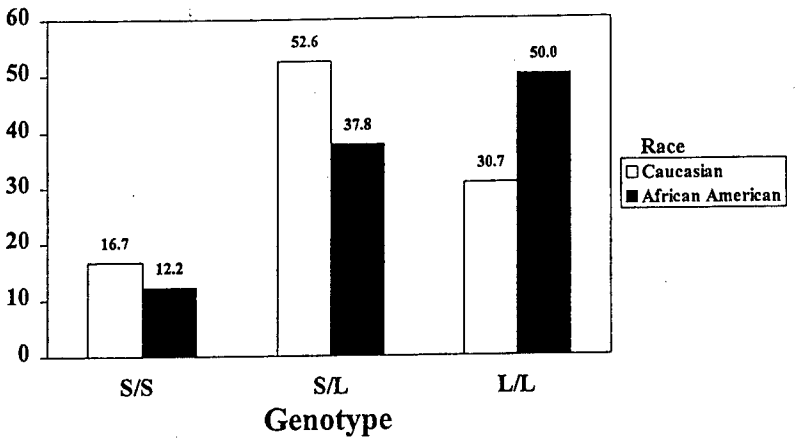

Fig. 1. Prevalence of *5-HTT* genotypes by race. $\chi^2(2)$, 10.6; *P*, 0.005; *S*, short variant of *5-HTT; L*, long variant.

newspaper advertisements and flyers in the metropolitan Washington, D. C., and Philadelphia areas for a free smoking cessation program. Nonsmoking controls (n = 230) who reported having smoked fewer than 100 cigarettes in their lifetimes were recruited through similar mechanisms. Exclusion criteria were as follows: under age 18, a personal history of cancer, undergoing treatment for drug or alcohol addiction, or presence of a psychiatric disorder that precluded informed consent.

Procedures. During a visit to the clinic, subjects completed an informed consent form reviewing the study procedures, including genotyping, and completed questionnaires assessing demographics and smoking history variables (age at smoking initiation, longest prior quitting period, and current smoking rate). Subjects then received a single, minimal contact (1 h) of behavioral smoking cessation counseling and self-help materials (17). They were followed for 1 year afer the counseling to assess self reports of quitting. The quitting outcome measure was a 30-day period prevalance of smoking 12 months after smoking cessation treatment. All subjects donated blood for genetic analysis.

Oligonucleotide primers flanking the 5-HTTLPR sequence (5′ggcgttgccgctctgaattgc and 5′-gagggactgagctggacaacccac; Ref. 7) from the *5-HTT* gene 5′-flanking regulatory region generating 484 or 528 bp were amplified by PCR using 50 ng of genomic DNA, 2.5 mM deoxyribonucleotides, 0.1 μg of primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgC12, and 1 unit of Taq DNA polymerase. Cycling conditions included melting at 95°C (5 min), 35 cycles of 95°C for 30 s, annealing at 62°C for 45 s, and an extension at 72°C for 1 min. A final extension at 72°C for 4 min completed the PCR. The amplified product was resolved by agarose gel electrophoresis (1.5%). The assay was validated by confirming polymorphic Mendelian inheritance patterns in seven human family cell lines (n = 134) encompassing three generations each (data not shown; National Institute of General Medical Sciences, Human Genetic Mutant Cell Repository, Coriell Institute, Camden, NJ). Twenty % of the samples were repeated for quality control.

χ^2 tests were performed to evaluate associations of *5-HTT* genotypes with ethnicity and with categorical smoking outcome variables (*i.e.*, smoker *versus* control; among smokers only: age at smoking initiation and 1-year abstinence). Fisher's exact test was used for the abstinence variable due to small cell sizes. One-way ANOVAs were performed to examine associations of *5-HTT* genotype with continuous outcomes in smokers (*i.e.*, longest prior quit period and number of cigarettes/day).

Results

The study sample included 280 (56%) females and 218 (44%) males. Of the 268 smokers, 221 (84%) were Caucasian and 47 (16%) were African American. Of the 230 nonsmoking controls, 203 (88%) were Caucasian and 27 (12%) were African American. The average age of study participants was 43.8 ± 11.5 years; 89% of participants had education beyond high school. Among the smokers, the average smoking level was 21.8 cigarettes/day.

As shown in Fig. 1, significant racial differences in the distribution of *5-HTT* genotypes were found (χ^2 = 10.6; P = 0.005). Caucasians were significantly more likely to carry the short variant of the *5-HTT* gene than were African Americans. Therefore, analyses of the associations of genotype with smoking practices were stratified by race.

The prevalence of *5-HTT* genotypes by smoking groups is presented in Table 1. There were no significant differences in the distribution of genotypes in smokers *versus* nonsmokers in either Caucasians or African Americans. Among smokers, we used χ^2 tests of associations of age at smoking initiation (<16 *versus* ≥16 years) and the 12-month posttreatment quit rates with *5-HTT* genotypes; no associations were found in Caucasians or African Americans. The *5-HTT* genotype was not associated significantly with the longest prior quitting period (in days) in either Caucasian smokers (n = 221; F = 0.14, P = 0.89) or African American smokers (n = 47; F = 0.43, P = 0.65). Nor was *5-HTT* genotype related significantly to current smoking (number of cigarettes/day) in Caucasians (F = 0.41; P = 0.66) or African Americans (F = 0.44; P = 0.64).

Discussion

The present case-control study was the first to evaluate whether or not smoking was associated with a polymorphism in the serotonin transporter (*5-HTT*) gene in Caucasians and African Americans. Previous evidence linking this polymorphism with anxiety (9) and supporting the potential benefits of serotonin reuptake inhibitors in smoking cessation (15) suggested that this gene may be a plausible candidate for predisposition to nicotine dependence. In this study, we found no evidence for associations of *5-HTT* with current smoking, smoking history, or cessation rates in either racial group. It should be noted, however, that subjects recruited through newspaper advertisements may not be representative of smokers and nonsmokers in the population. Nevertheless, these findings suggest that the serotonin transporter gene polymorphism studied here

Table 1 Prevalence of *5-HTT* genotypes within smoking groups by race

Group	Caucasian			African American		
	S/S[a]	S/L	L/L	S/S	S/L	L/L
Smokers, *n* (%)	43 (19.4)	108 (48.9)	70 (31.7)	5 (10.6)	18 (38.3)	24 (51.1)
Controls, *n* (%)	28 (13.8)	115 (56.6)	60 (29.6)	4 (14.8)	10 (37.0)	13 (48.2)
χ^2	3.40			0.28		
P	0.18			0.87		
Smokers, starting age <16 yr	17 (22.7)	38 (50.7)	20 (26.6)	1 (7.7)	5 (38.5)	7 (53.8)
Smokers, starting age ≥16 yr	26 (17.9)	69 (47.6)	50 (34.5)	4 (11.8)	13 (38.2)	17 (50.0)
χ^2	1.61			0.17		
P	0.45			0.92		
Smokers, quit at 1-yr follow-up	6 (22.2)	12 (44.5)	9 (33.3)	0 (0)	2 (50.0)	2 (50.0)
Smokers, smoking at 1-yr follow-up	27 (18.6)	73 (50.3)	45 (31.0)	1 (4.4)	9 (39.1)	13 (56.5)
P[b]	0.85			1.00		

[a] S, short form; L, long form.
[b] Fisher's exact test.

is not a major determinant of cigarette smoking practices. Further investigation of other polymorphic serotonergic genes, such as those regulating postsynaptic receptor function, are needed to fully evaluate the role of serotonin transmission in smoking behavior.

As in our previous studies of dopaminergic genes, we found evidence for significant racial variation in genotype frequencies. In the present study, Caucasians were significantly more likely than African Americans to carry the short variant of *5-HTT*, which has been associated with anxiety-related traits (9). However, the sample size of African Americans in our study was small. Previously, we found racial differences in the frequencies of the dopamine D2 receptor (*DRD2*), and dopamine transporter (*SLC6A3*) genes.[2] In both cases, African Americans were significantly more likely to have genotypes associated with reduced dopamine transmission. Evaluation of racial differences in the frequency of genes governing neurotransmitter function may enhance our understanding of genetic contributions to race differences in smoking practices (18).

To fully elucidate the influence of genetic factors in cigarette smoking, it will be necessary to examine the interplay of the genes involved in synthesis, release, and receptor function for a variety of neurotransmitters. Examination of genetic factors in nicotine metabolism may also be fruitful. A better understanding of these pharmacogenetic mechanisms can lead to the development of improved prevention and treatment strategies tailored to the needs of individual smokers.

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